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Host Signaling Response to Adhesion of Bifidobacterium infantis

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HOST SIGNALING RESPONSE TO ADHESION OF *BIFIDOBACTERIUM INFANTIS*

by

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ABSTRACT

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Investigations of the molecular binding partners of the probiotic bacterium *Bifidobacterium longum* subspecies *infantis* (*B. infantis*) and the pathogen *Salmonella enterica* subspecies *enterica* serovar Typhimurium LT2 (*Salmonella* ser. Typhimurium*)* found that these two very different bacteria bind gangliosides. However, these organisms lead to completely different host health outcomes when present in the gut. *B. infantis* is the founding microbial population in the intestinal tract of breast-fed infants. *S. typhimurium* is the most important food-borne pathogen that results in humans. This study used an *in vitro* gut epithelial cell model to examine the host cellular response to adhesion of *B. infantis*, which led to an increase in intestinal epithelium survival. This observation led to a series of experiments to elucidate the pathway for host signaling initiated by

adherence of *B. infantis* to the host membrane to explain the increase in host cell survival. *B. infantis* adhesion induced significant (q≤0.05) differential expression of 208 host genes. These genes were associated with increased broad mechanisms of cell survival that included BIRC3, TNFAIP3, and SERPINB9. We hypothesized that a biochemical link existed between the host membrane adhesion protein and the increase in cell survival, mediated via AKT. We tested this hypothesis to demonstrate that *B. infantis* interaction initiated signal transduction using G-proteins via phosphorylation of AKT and induced production of the BIRC3, TNFAIP3, and SERPINB9. This study discovered adhesion of *B. infantis* initiated activation of AKT via phosphorylation of both Ser473 and Thr308, which results in increased cell survival.

(131 pages)

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ABBREVIATIONS

BSA..... Bovine serum albumin

C(t)..... Threshold cycle number

DAG..... diacylglycerol

DMEM..... Dubelco modified Eagle minimum essential media

ECM..... Extracellular matrix

FBS..... Fetal bovine serum

FDR ..... False-discovery rate

GALT ..... Gut-associated lymphatic tissue

GCOS ..... GeneChip operating Software (Affymetrix)

GO ..... Gene ontology

GPCR ..... G-protein coupled receptor

IBS.....irritable bowel syndrome

IP3 .....inositol triphosphate

IPA.....Ingenuity pathway analysis

LPS.....Lipopolysaccharide

M-cells .....Microfold cells

MOI.....Multiplicity of infection

MOPS .....3-morpholinopropanesulfonic acid

MRS.....deMan, Rogosa and Sharpe medium

OD_{600} ..... Optical density at 600 nm

qPCR .....Quantitative polymerase chain reaction

PBS..... Phosphate buffered saline

PIP3..... Phosphoinositol tri-phosphate

PLC..... phospholipase C

PVDF..... Polyvinylidene fluoride

RMA..... Robust multichip average

RT-PCR..... Real-time polymerase chain reaction

SAM..... Significance analysis of microarray

SDS.....Sodium dodecyl sulfate

TBS..... Tris base and sodium

TTBS.....Tris base sodium and tween 20

TC.....Total cell count

TES..... N-(Tris(hydroxymethyl)methyl)-2-aminoethanesulfonic acid

TLR..... Toll-like receptor

VC.....Viable cell count

CHAPTER I

INTRODUCTION

The science of microbiology and immunology, among others, owe their origins to studies investigating the interactions between humans and pathogenic microbes (135). The bulk of knowledge concerning the molecular interactions between eukaryotic and prokaryotic organisms is largely restricted to infectious disease agents and their impact on the human condition. However, throughout the decades increasing amounts of information about the interactions between humans and bacteria that do not result in disease is gaining importance with the rapid increase in discoveries that describe the important contribution of commensal and probiotic bacteria to human health and chronic disease (53).

The human gastrointestinal tract is home to ~10 times more bacterial cells than the number of somatic and germs cells in the entire human organism, which live harmoniously and in some cases provide health benefits to their human host (129). In contrast, the link between gut microbiota composition, enzymatic activity, and dynamic nature with human health is being associated with specific diseases, including arthritis, cancer, and obesity (27, 88, 143). Consequently, the gut microbiome project, MetaHIT, an international collaboration, was initiated to define the gut microbial community structure of humans around the world. The goal of this initiative is that it will define normal and aberrant gut flora in a concerted effort to adequately describe the links between microbes and the human condition (38, 53).

The microbial community structure of this complex ecosystem is classically divided into parts pathogenic and commensal microbes (61). It is recognized to contain an extremely complex set of microbe groupings that play different roles depending on the host condition, immune status, and the results of interactions between the gut microbiota and the host. More recently, specific bacteria are being correlated to improved immune system function, gastrointestinal balance, lower incidence of diarrhea and other specific benefits that they deliver to the host (117). These so-called probiotic bacteria, which are defined as organisms that upon consumption yield the host health benefits beyond simply providing the host with nutrition (94), are now commercially available in foods that claim to increase gut health, increase regularity of bowel function by reducing intestinal transit time, and improve immune function. They have long been advocated by Mechnikov (148) who claimed they could increase longevity for those who consume fermented milk products containing these microbes. Unfortunately, until recently the specific and repeatable health benefits of probiotic microbes were more fiction than reality (6, 12). In the last few years, molecular interaction studies yielded specific molecules and mechanisms that probiotic microbes induce in the host during consumption (40, 45, 140, 155). Considering the vastly different health effects that different bacteria can provide to a host, more detailed and mechanistic investigations must brought to bear to determine what underlies the claimed probiotic benefits so that specific traits and known biology can be directly linked to specific host benefits.

Like pathogens, probiotic bacteria must associate with the host via cellular interactions to bring about their beneficial changes (29). Sela et al. (133) defined an example of these beneficial interactions in the microbial colonization of human infant guts via selective growth enrichment via consumption of the complex oligosaccharides found in human breast milk. This leads to a rapid expansion of *B. infantis* promoting infant gut colonization by this microbial population in breast-fed infants (81). This organism contains a specific set of ~20 genes needed to consume the diverse and complex oligosaccharides that are found in human milk. Presumably, selective growth advantages of *B. infantis* will lead to additional adhesion and health modulation in the infant. Consequently, adhesion is a critical step in host/microbe association. Among other actions, adhesion enables an intimate connection between two very different cells that likely initiates molecular signal transduction responses in both cells that leads to extensive changes to the gut epithelium as well as other tissues depending on the cognate receptor interactions.

In the mammalian gut there are many different cell types that includes epithelial cells, as well as gut-associated lymphoid tissue (GALT), Peyer's patches, isolated lymphoid follicles, microfold cells (M-cells), dendritic, and immune cells (B- and T-cells) (42, 104, 106). While an extensive amount is known about how pathogenic bacteria invade host cells (43, 66, 83, 121, 132) relatively little is known about the molecules that are used to bind the host cell that initiates the complex set of interactions used to impart their action to the

host. Unfortunately, very little is known about how probiotic bacteria associate with host cells, yet they clearly have a role in gut health through out life.

Eukaryotic cells commonly transduce external signals into communication mechanisms via protein-protein interactions, small molecules crossing the cell membrane, and glyco-conjugate interactions (109). Each of these signaling methods can be activated by the adhesion of a bacterial cell to a host cell (66, 68). Host signal transduction resulting from bacterial adhesion likely evolved from different and multiple adhesion molecular events and may account for various phenotypic reactions displayed by the host, which includes well over 100 molecules each of which lead to multiple and interconnecting signaling networks inside the host cell to change the phenotype. While only a few representative members of the interaction types have been explored well enough to define the specific molecules that interact between the two cell types, let alone the different types of microbes and host cells, these studies are largely focused on host/pathogen interactions the lead to disease. Due the disease state and the impact to human health, the molecular interactions of infectious disease agents has largely focused on invasion and induced cell death (i.e. apoptosis) (43, 77, 113, 121). These studies led to discovery of toll-like receptors, clatherin-dependant endocytosis, chemokines, and other key host signaling pathways that explain disease symptoms and the molecular progression of infectious diseases all with the aim to reduce or mitigate the symptoms (86, 122, 156).

Recently Desai *et al.* (31, 32) demonstrated that probiotic and various pathogenic bacteria bind gangliosides covalently bound to solid glass beads. These are the same component of the mammalian extra cellular matrix (ECM) and are binding partners of various molecules and pathogens, including *Salmonella* ser. Typhimurium, *Salmonella enteridis, Escherichia coli, Clostridium botulinum* neurotoxins (142), *Haemophilus influenzae* (22) and many viruses (141). However, this observation leads one to ask many intriguing questions:

- If two very different bacteria are binding the same component of their host cells, what accounts for the difference in the host reaction?
- What changes are induced by probiotic adhesion in the presence of a pathogen?
- Can molecular binding events account for claimed health benefits of probiotic bacteria?
- What signal transduction routes are changing that lead to a non-pathogenic outcome?

These questions and preliminary data led to the hypothesis that the signaling events resulting from host cell adhesion of *B. infantis* induce gene expression changes associated with longevity and promote cell survival. Describing the molecular mechanism that results in health benefits for the host is required for the identification of other potential probiotic bacteria as well as the application of probiotic microbes as medical treatments for specific diseases (117). Recent clinical studies report benefit for treatment of irritable bowel syndrome using

B. infantis (152). As *B. infantis* is not a pathogen, where nearly all of the knowledge related to host signaling related to host-microbe interaction lies, there is little to no molecular mechanistic explanation for the reported benefits.

This study examined the hypothesis by characterizing the host signal transduction networks induced by adhesion of a known probiotic bacterium, *B. infantis*, to gut epithelial cells *in vitro.* By determining the host gene expression a potential pathway was determined and subsequently validated using western blot analysis for protein level increases and phosphorylation changes that lead to activation of specific transduction pathways to prove the molecular mechanism leading to decreased cytotoxicity.

CHAPTER II

LITERATURE REVIEW

Bacterial Adhesion Mechanisms

The varied means that bacteria use to adhere to surfaces in environmental niches is as diverse as the bacteria themselves. Adhesion to human cells is the initial necessary step for bacteria to colonize, bind cellular molecules, and leads to invasion. As such the mechanisms of pathogenesis has been examined in detail (11, 35, 43).

The structures described in detail that pathogenic bacteria use for adherence to host cell membranes consist of fimbriae or pili (113), bacterial lectins (91), flagella (107), and lipopolysaccharides (LPS) (76) (TABLE 1). In all cases these bacterial structures are defined to bind sugars or complex sugars on the host. These adherence factors along with all other proteins that are specific to bacterial adhesion to some extracellular surface are collectively known as adhesins (77). Specifically, these attachment factors are necessary to help the bacteria overcome the net negative charge that exists on both the host cell and the microbial cell (113). Unfortunately, the proteins that are used to mediate other interactions beyond sugars are yet to be fully described.

Fimbriae or pili are hair-like structures that extend out from the surface of bacteria. Often found along the edges or tips of the pili are specific proteins that bind to specific host targets (35). Fimbriae are found in both Gram-positive and

TABLE 1. Classes of adhesins used by pathogenic bacteria.

Adhesin	Structure	Bacterial Function	Host Target
Fimbriae (pili)	Short hair-like structures, often hundreds cover entire surface of organism, 0.5-10 μM in length	Transfer genetic material and or proteins during conjugation	ECM components, surface receptors, use glycan (mannose) to bind
Bacterial Lectins	Protein or protein complexes, located on bacterial surface, or at ends of pili and flagella,	None known; only observed in specific protein-saccharide interactions; thought to bind oligosaccharides during adhesion to surfaces	Saccharides-found on glycolipids, glycoproteins, or other host structures. Often specific for mannose
Flagella	Long, flexible hair-like structures, can be localized to one region, few in number, up to 15 μM long	Provide motility	Surface receptors, bacterially excreted proteins, ECM components, use glycan (mannose) to bind
LPS	Lipid-A base, saccharide core with O-antigen terminus. Typically anchored in cell surface but can be excreted	Component of Gram-negative cell wall.	TLR4 & 5, host lectins

Gram-negative bacteria, and are separated into different categories or types based on how the structures are assembled (113). For gram-positive organisms there are two main classifications - rod-like and flexible (46, 113). In Gram-negative bacteria five types of pili are found: 1) chaperone–usher pili, 2) curli, 3) Type IV pili, 4) Type III secretion needle (part of the Type III secretion system that delivers effector molecules to invade the host), and 5) Type IV secretion pili. As noted in the name of some of the pili classes, these structures are not only used for adhesion, but also for the secretion of proteins and genetic material during conjugation, as well as motility (46, 70). The adhesion of *Salmonella* sv Typhimurium to specific cell types when expressing specific fimbriae is a prototypical example of how fimbrial structures allow for specific binding between host and microbe that are tissue specific and regulated by the local environment (9). In *Salmonella* infections a fimbrial lectin, SEF17, specifically binds to host epithelial fibronectin (an ECM glycoprotein) allowing for the bacteria to adhere and initiate the infection process with the host cell via subsequent endocytosis mechanisms (80, 82, 105, 118).

Lectins are proteins that specifically bind saccharides (90). Specificity is sufficiently high so as to discriminate D-glucose-binding and D-mannose, the C-2 epimer of glucose (39). Nearly all organisms express proteins with lectin activity (91, 93) and are involved in host-host, host-virus, and host-microbe interactions (91). Lectins are often found as integral parts of other bacterial adhesins, such as pili, and may account for the adhesive properties of those structures (91). Lectins

are often large multi-domain protein complexes but the actual carbohydrate binding activity is usually observed in a single protein sub-unit or domain within the larger molecular complex (91). The most studied case of lectin mediated host-microbe interactions is that of host mannose, galabiose, and N-acetylglucosamine binding by lectins produced by *E. coli* (107, 147). *E. coli* P-type fimbriae specifically bind galabiose found in human urinary tract tissue; their S-type fimbriae bind mannose in human neural tissue, and *E. coli* K99 fimbriae bind N-acetylglucosamine found in intestinal cells (147).

Flagella are long, flexible protein structures that are responsible for bacterial motility. They are also implicated, as in the case of fimbriae, to display lectin activity, which account for their role in mediating microbial adhesion (35). Enterotoxigenic *E. coli* flagella and its adhesion to host cells is the most studied model with respect to flagellar-mediated adhesion to the gut. In this model, the bacterium secretes a protein, EtpA, which is bound at the end of the flagella filament. The flagellar complex bound by EtpA is presented to host that leads to adhesion between host and microbe (124). Flagellin, the monomeric constituent of bacterial flagella, has long been known to induce an inflammatory response via the binding of the host immune receptor TLR5 (51). Due to the fact that flagella are used primarily for motility and that the mammalian immune system has ample receptors specific to bacterial flagellin, initially the thought that flagella also mediated adhesion was somewhat controversial; in that light that a system that evolved two separate mechanisms for adhesion and motility would seem

beneficial (43, 98). In support to dual systems, *Salmonella* spp. secrete flagellin to specifically elicit an inflammatory response and thereby recruit the dendritic cells, the cell that contains the *Salmonella* specifically target (15). It is no longer in question that flagella mediate glycan-linked adhesion in eukaryotes (107, 119). Lipopolysaccharides (LPS, aka endotoxin) mediate adhesion between bacteria and host cells using Toll-Like Receptors (76, 154). LPS is a component of Gram-negative bacterial cell membrane and also acts as a toxin when independent of the bacterial cell (115). LPS (Fig. 1) consist of a lipid A base, which largely contributes to the toxic nature of this molecule, a saccharide core and an O-antigen terminus (146). LPS elicits a rapid and sever inflammatory response in healthy mammals, which is initiated by a chemokine signaling cascade initiated by TLR4, which specifically binds LPS during the infection process (154).

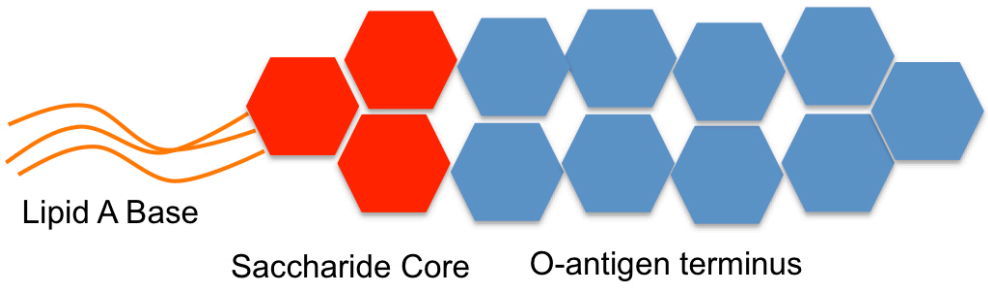

FIG. 1. Schematic representation of LPS. The lipid base, shown as orange lines, is often associated with the bacterial cell membrane with the O-antigen, shown as blue hexagons, pointed away from the bacterial cell. The saccharide core, shown in red, is often composed of oligosaccharides such as heptose.

Bacteria evolved many ways to adhere to host cells. Definition of specific molecules involved in the association and the signal transduction beyond sugars and glycans is lacking. This area is growing due to the need for new antibiotics and emergence of multi-drug resistant strains in gut pathogens, especially *Salmonella* (19). However, an extensive amount of work is done to define the host response to bacterial attachment beyond the use of glycosylation or polysaccharides.

It should be noted that *B. infanits* is non-motile (23) or produce LPS. Without these mechanism commonly used by other bacteria to modify host cell signaling, the question as to how *B. infantis* modifies host cell signaling remains.

Microbes and Host Cell Signaling

Successful microorganisms respond to external stimuli by producing a reaction that results in an increased chance of survival, which leads to adaptation and evolution of specific responses due to multiple environments and conditions. Multicellular organisms face an extra level of complexity in responding to external stimuli because it requires that individual cells communicate with the other cells at distant locations to produce a coordinated response. Additionally, multiple cell types within the organism produce and respond to many signals simultaneously. Transmission of the environmental stimulus to the interior of the cell for communication is known as cell signaling or signal transduction. The mechanisms of eukaryotic cell signaling have evolved over time into elegant and complex networks, with multiple levels of sensitivity, specificity, and regulation.

Despite the diverse means of signal transduction that have evolved there are some common themes that are used within a network to channel chemical signals into cellular responses.

There are two main levels of cell signaling - external stimuli mediated by ligands binding cellular receptors to propagate a signal into the cell and intercellular communication, which begins with production of molecules that interact with other cells. These are key to rapid changes in individual cells to encompass the entire multicellular organism. Communication occurs when a signal is propagated through the network to the appropriate target molecules so that a response can be mounted by the cell (110). Once transduced inside the membrane, signal transduction is further perpetuated by the use of several strategies that include modification of signaling molecules with sugars, phosphates, fatty acids, and additional carbon group modification (e.g. methylation, acetylation). An example of this type of communication (Fig. 2) is the activation of G-protein coupled receptors (GPCR). Often this interaction is a combination of a non-specific adhesion factor that brings the organism close to the cell that is followed by a more specific protein/cell interaction. Viruses use this strategy to gain entry into host cells (127). In some cases glycolipids (e.g. gangliosides) are the non-specific binding partner that leads GPCR activation (99). Another, example is where an enzyme, phospholipase C - a known bacteria-associated virulence factor, directly activates and transduces virulence via membrane GPCRs (24). Subsequently, this lipase cleaves PIP3 into two

additional messenger molecules, IP3 and DAG, both of which cause a series of additional signaling changes in the cell that lead to transcriptional changes (20).

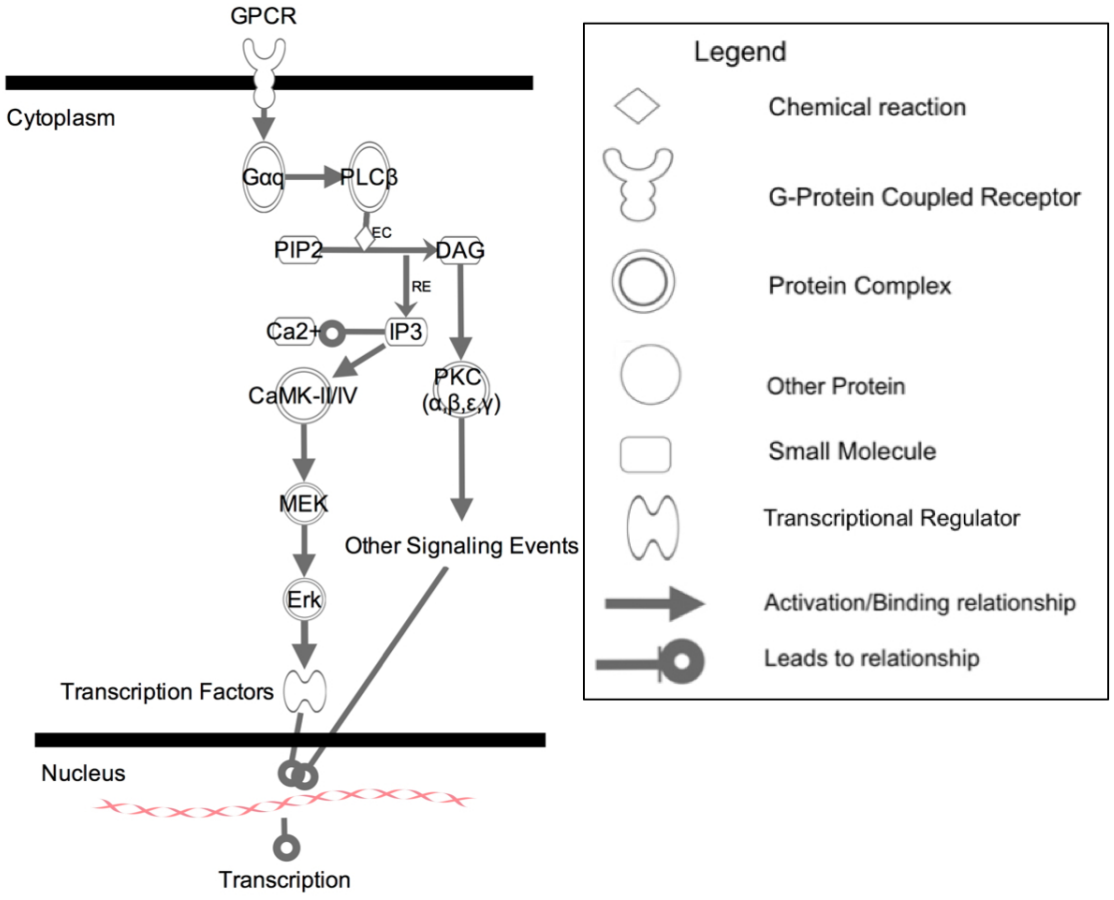

FIG. 2. GPCR signaling pathway involving phospholipase C. GPCRs activate phospholipase C (PLC), which initiates signaling cascades leading to transcriptional changes and virulence with bacterial adhesion.

This is the most prototypical GPCR signaling pathway. In this manner an external stimulus is integrated and results in a signal being perpetuated inside a cell to cause a specific response attributed to a specific biological event at the cellular membrane.

The other main level of signaling occurs between cells. Intercellular signaling can result from the interaction of surface-bound receptors with a product from another cell type. For example, chemokines and cytokines are immune-modulating molecules produced by immune cells to recruit additional cells to a specific location. These molecules are bound by receptors at the surface of migrating leukocytes that causes these cells to take on new activities or migrate to a new location via concentration gradients (153). Other examples include signaling molecules that bind surface receptors, like GPCRs, to modify cellular transcription (44), or cross the cellular membrane to receptors that lie within a cell such as nitric oxide (30).

Intercellular signaling can take place over large distances. Many different types of signaling molecules ranging from small soluble chemicals to large proteins (e.g. hormones) or fatty acids travel between cells and organs to induce changes in the organism. These molecules include gases like nitric oxide, peptides such as insulin (74), small molecules such as Ca^{2+} ions (74), fatty acids, and fatty acid derivatives, such as prostaglandins (7), along with many other examples.

Another example is cell-to-cell communication mediated by contact and adhesion via proteins expressed on cell surfaces that initiate signals in neighboring cells (Fig. 3). Signaling molecules that are bound to cell surfaces are effective at perpetuating a signal when that cell comes in contact with another cell that is displaying the appropriate receptor.

The different properties of each signaling molecule allow for greater diversity of signaling channels, receptor specificity and sensitivity, as well as means of regulation. The combination of all of these factors allow for a near limitless variety of signals that a cell can produce and process.

For each signaling molecule there is a receptor that can detect the presence of the signaling molecule and in some manner perpetuate the signal. My hypothesis is that *B. infantis* is acting as a signaling molecule setting off an undefined signaling cascade leading to improved host health. As *Bifidobacterium* spp. do not invade host cells (103), yet do induce changes in host cells we will examine the largest classes of cell surface receptors (Fig. 3) that are ion channel-linked receptors, G-protein coupled receptors, and enzyme linked receptors (1). These receptors are of interest because it has been demonstrated that bacteria can act as signaling molecules, binding the surface of host cells and initiating signaling cascades and they are co-receptors for gangliosides (66, 132).

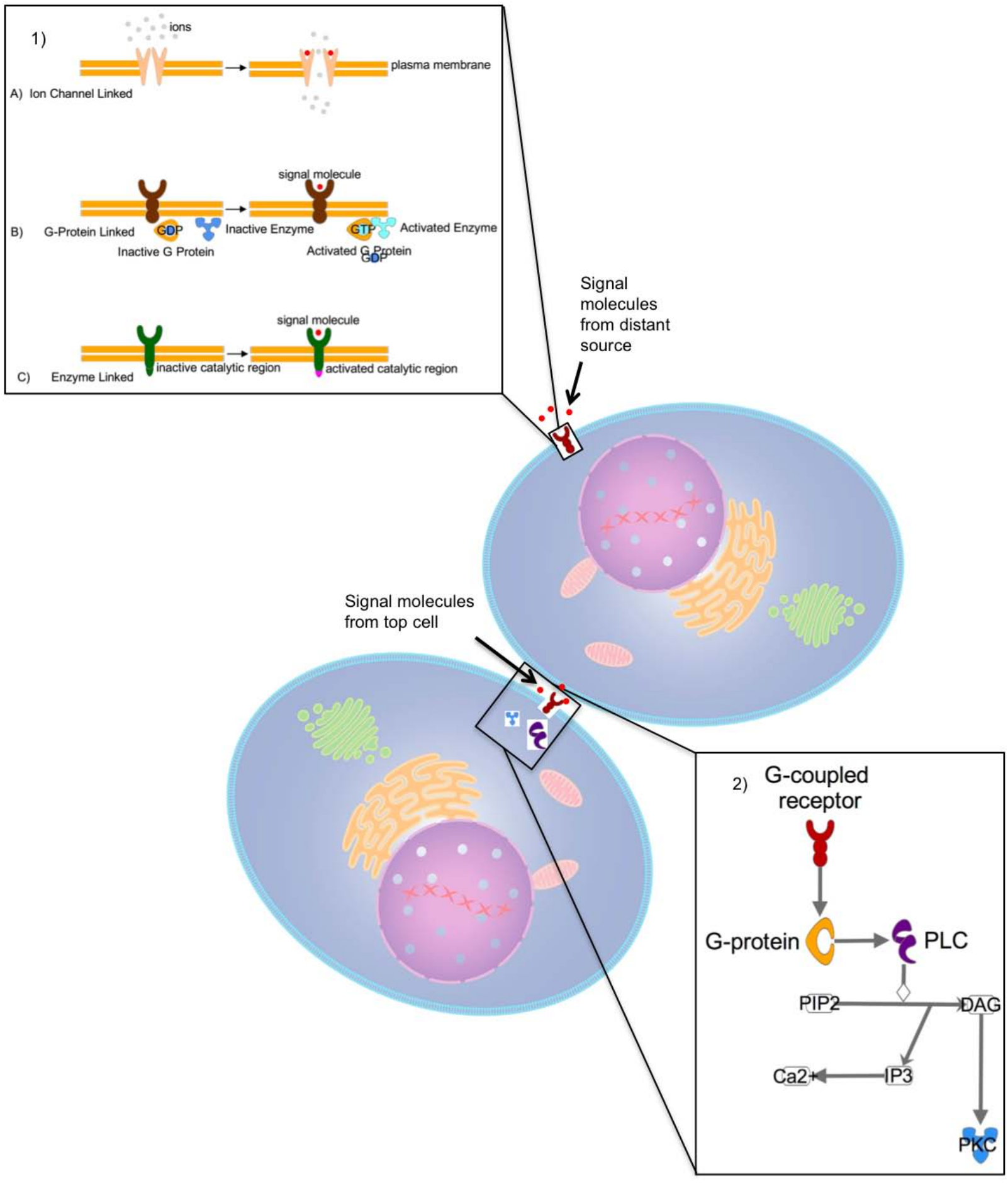

FIG. 3. Eukaryotic cell signaling induced by channels and G-proteins. Panel 1 depicts Intercellular signals are often internalized using these common surface receptors. Example A depicts an ion channel-linked surface receptor responding to ligand binding by opening ion specific channels that allows ions to move into or out of a cell (2). Example B demonstrates a G-protein-linked surface receptors upon ligand binding activate the associated G-protein. Inactive G-proteins bind GDP. Upon activation the GDP is displaced by GTP, allowing the G-protein to activate its target enzymes (111). Example C shows an enzyme-linked surface receptor that bind a ligand to catalyze a reaction that perpetuates an intracellular signal (131). Panel 2 is an example of intercellular signaling pathway.

Extra Cellular Matrix, Gangliosides, and Host Signaling

Microbial binding structures have evolved to bind to a variety of host structures proteins in the extracellular matrix (ECM) (31, 54) and others as previously mentioned (14, 39, 64, 71, 91, 121). These receptors are often found as components of or linked to the host cell signaling machinery, and as such bacteria, and bacterial secreted components, become signaling molecules. It then becomes of great interest what the host signaling response is to the adhesion of different bacterial species. With the intense focus on the sugar-binding molecules, exploration and definition of the binding partners between the bacterium and the host for the other class of molecules is largely unexplored.

As a common site of bacterial adhesion and of special relevance to this work is the mammalian ECM (123). The ECM is a structure of proteins and polysaccharide macromolecules that are largely responsible for cell and tissue differentiation, migration, repair, adhesion and even wound healing (59). The overall form and function of tissues is largely a result of cellular ECM interactions (49, 108). Major constituents of the ECM are glycosaminoglycans (largely found attached to proteins resulting in the formation of proteoglycans (57)), and fibrous proteins including collagen (69), elastin, fibronectin, laminin, and vitronectin (136), all of which are highly glycosylated (147). This results in a myriad of niches, motifs, and structures that are ideal for bacterial adhesion to occur via fimbriae or other proteins on the bacterial surface (11). This matrix also plays a key role in processing cellular communication and tissue structure. The ECM

binds cells to the basement membrane via integrins in the membrane, which is also involved in cell shape with the intimate association with actin in the cytoskeleton (128).

Integrins are signaling proteins that are responsible for binding and responding to stimuli originating in the ECM, and can perpetuate signals between cells (17, 52, 63) that enable the ECM to facilitate eukaryotic cell signaling events between the epithelium and the other underlying tissues (128). Some pathogens, such as *Shigella flexneri,* some *E. coli* strains, and some *Salmonella* serovars bind integrins to hijack the host signaling mechanisms that perpetuate invasion and subsequent systemic infection (73). The exact binding domain (glycan or protein) is not yet defined.

Gangliosides are key components of the host plasma membrane that play integral roles in signaling (55, 97, 99) and are constructed of a ceramide core with an attached oligosaccharide (together called a glycosphingolipid) to which one or more sialic acid is bound (78). These compounds are found in high concentration in lipid rafts, where interestingly GPCRs are also found to be concentrated. Maudsley *et al.* (97) found that when GPCRs are located in lipid rafts they initiate cell signaling networks that lead to increased cell proliferation. In other cases in the same cell GPCRs outside of lipid rafts initiate signaling networks that lead to decreased proliferation. Further links between GPCR signaling and gangliosides was described by Gouni-Berthold *et al.* (55) whose work showed that gangliosides initiated signaling events via GPCR pathways in

smooth muscle cells. McNamara *et al.* (99) described how *Pseudomonas aeruginosa* flagella bind gangliosides *in vitro*. Close association of gangliosides with host cell signaling events, specifically via GPCRs, and the known binding of gangliosides by *B. infantis* (31, 32) recently demonstrated by Desai *et al.* point one to question. Does *B. infantis* initiate signaling cascades via GPCRs following adhesion of gangliosides?

Bacteria modulate host cell signaling during adhesion and invasion (66, 73, 132). *Yersinia* infections are a classic example of how a pathogenic microbe affects the signaling mechanisms of a host cell. As reviewed by Isberg *et al.* (66), a protein expressed on the surface of *Yersinia spp.* called invasin is able to bind multiple integrins that are expressed on the surface of microfold cells (M-cells) found in Peyer's patches. These specialized cells are responsible for antigen sampling in the lumen of the gut and are considered to be the front line of the mucosal immune system (67). Integrins, the molecular binding partner of invasin, are responsible not only for cellular adhesion but also initiating intracellular signaling cascades (17, 52, 63). By specifically binding integrins found on M-cells *Yersinia spp.* induce M-cells to phagocytose the bound bacterium by manipulating the host signaling mechanisms. Subsequently, the internalized bacteria are hidden within a membrane bound compartment inside the host cell preventing the organism's immune system from eliciting a response to eradicate or clear the infection.

Another example of cell signaling manipulation by a pathogen is *Listeria monocytogenes* gut infections. Similar to many gut pathogens, *L. monocytogenes* induces phagocytosis by manipulating the host signaling mechanisms for the cytoskeleton. This organism uses internalin A and B, which interacts with E-cadherin on the host membrane to induce cytoskeletal changes that enables endocytosis and access to the cytosol (132). In the host, E-cadherin is a key protein that mediates epithelial cell adhesion and tight gap junction maintenance, but also plays a role in intracellular signaling via the E-cadherin/PKC signaling pathway (79, 87, 145). *L. monocytogenes* uses this strategy along with actin movement to move laterally between cells as a method to evade the immune response so the infection can spread throughout the host. This strategy is also used to move between cell types. For example, this is how *L. monocytogenes* moves from the gut into circulation and then to cross the blood-brain barrier to cause a secondary infection from the initial invasion site in the gut epithelium (132).

Commensal bacteria also modulate the host immune system to allow the host to tolerate their presence (5). Recent findings linked host cell signaling caused by the presence of commensal bacterial LPS, which is usually only found on pathogens, and the subsequent decrease in the amount of pro-inflammatory mediators *in vitro* (36). Furthermore, factors produced by the host following the introduction of commensal bacteria initiate differential gene expression in the host with respect to cell surface receptors that are specific to bacterial surface

molecules, without which the cells ability to illicit an inflammatory response is greatly reduced (16). In effect, the commensal microbes prime the immune system. Additionally, *Bacteroides thetaiotaomicron* reduces inflammatory cytokine production by inducing nuclear exportation of RelA via a PPAR-γ-dependant pathway (72).

As previously mentioned, the vast majority of knowledge with respect to host microbe interactions is limited to the host-pathogen and host-commensal relationships (Fig. 3). In a recent review Marteau *et al.* (95) described many studies that demonstrated the results of the presence of probiotic microbes on host health, but noted that the field lacks specific information about the pathways from which those effects were derived. Even studies that have specifically addressed an individual probiotic microbe and the effect that specific microbe has on host health, have not demonstrated the specific mechanism of action, with respect to host signaling cascade or host-microbe binding partners (89). One study using *B. infantis* showed increased phosphorylation of MAPK proteins, but not the signaling route that led to increased phosphorylation or what phenotype the modified phosphorylation led to in the cell (40). Additional information to define the specific events that occur immediately following probiotic adhesion and the host signaling cascade that is triggered would provide critical information to provide a sound physiological role for these organisms. Furthermore this will aid in identification of new probiotic strains, as well as the implementation of probiotics to treat specific human ailments.

Bifidobacteria and Other Probiotics

What today are recognized as organisms from the genus *Bifidobacterium* were first described by Tissier in 1899-1900 and called *Lactobacillus bifidus* (112). The name was largely given due to frequent observation of bifurcating, gram-positive rod morphology observed in microscopy. Subsequent studies showed that this bacterium dominated the gut microbiota of breast fed infants (41, 151). Differences in *Bifidobacterium* populations depending on the food source of the infant were observed and studied in the early 20th century starting with Tissier, and continue to this day (75). Most recently the genome of *B. infantis* 15697 revealed genes that allow this bacteria to digest human milk oligosaccharides that remain indigestible to humans alone that give bifidobacteria a nutrient-based selective advantage in breast-fed infants (133).

Probiotics are bacterial organisms that when consumed provide some benefit to the host other than the basic nutrition gained from their consumption (47). The term “probiotic” was most likely first used by Kollath in 1953 to describe anything that restored the health of patients suffering from malnutrition; however, direct association to bacteria came from Vergin slightly later (56). The idea that bacteria could have beneficial impacts on human health dates back to the Nobel Laureate, Ilya Ilyich Mechnikov, who later in his life recognized that by manipulations of the body’s microbiota by diet, the health of an individual could be influenced, which he quietly associated with benefit (148).

Recent clinical trials assessing the ability of a *B. infantis* strain to remediate the symptoms of irritable bowel syndrome (IBS) revealed that indeed giving encapsulated *B. infantis* 35624 to women with IBS significantly reduced their symptoms versus a placebo (152). This result most certainly classifies *B. infantis* spp. as a probiotic organism. While the beneficial aspects of these bacteria are known, the mechanisms by which these benefits are gained remain unclear (Fig. 4).

Numerical dominance of *B. infantis* in the infant gut of breast fed babies is well known (41, 75, 151). This near complete occupation of host gut at such a critical time in the organism's development strongly suggests an extended evolutionary history between the two organisms. If both organisms did not experience some positive influence towards their survival such a relationship would be selected against. This idea is further bolstered by the work of Sela *et al.* (133) showing that *B. infantis* contains genes that allow the bacteria to digest complex oligosaccharides found in human milk that are not able to be digested by human cells. While this could be the driving selective pressure that maintains this near symbiosis in infants, the mechanisms within the host that permit the bacterial presence, as well as the observations of improved health in adults, are not explained by Sela's observations.

Competitive exclusion of bacterial pathogens is often cited as the mechanism by which adult hosts gain benefits, as studies with other probiotics have demonstrated (85). Other groups found that certain *B. infantis* species

secrete soluble antimicrobial compound(s) that lead to the death of bacterial pathogens *in vitro* (89).

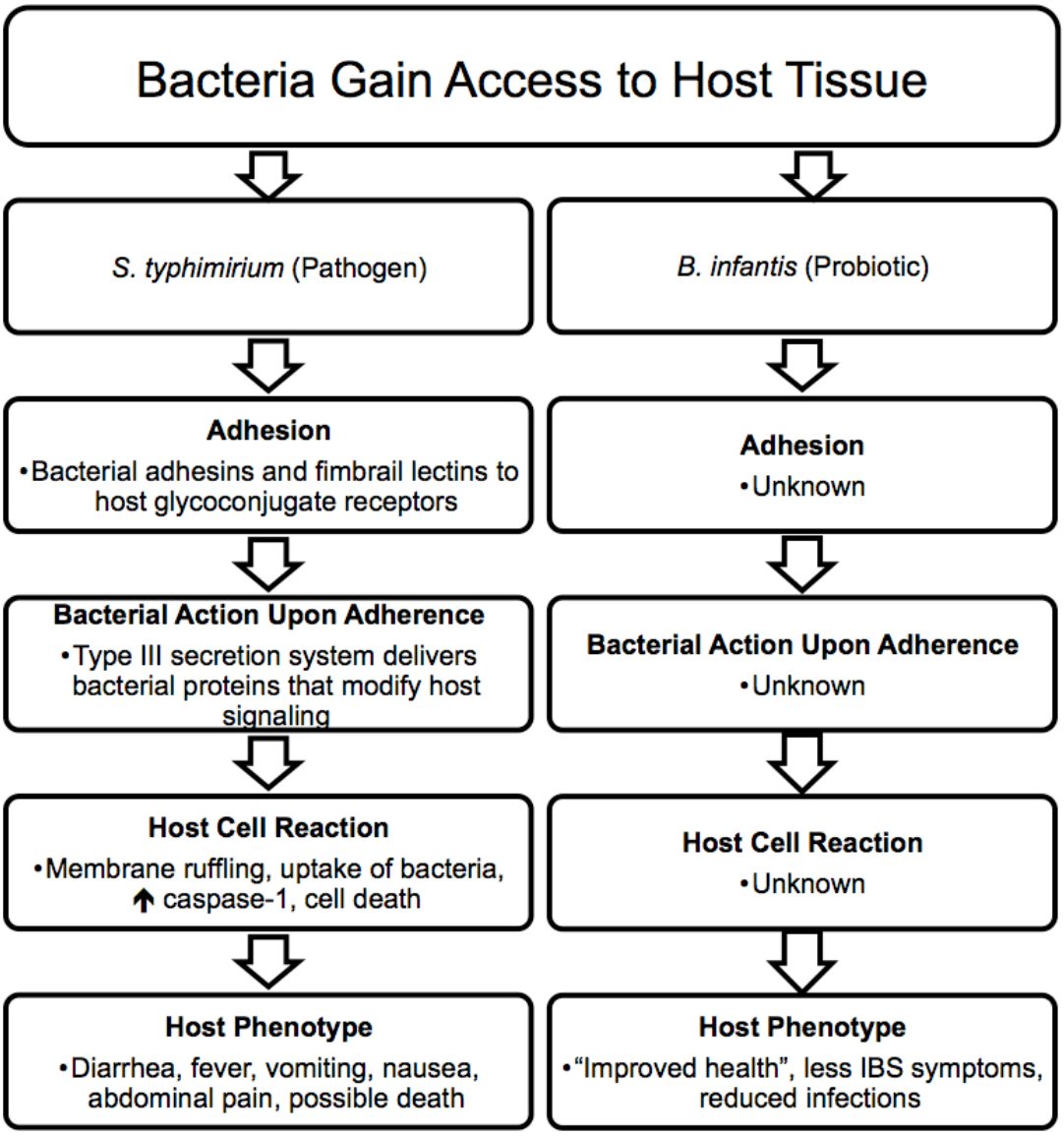

FIG. 4. Host colonization by pathogenic and probiotic bacteria.

While these observations may be contributing factors they completely ignore the most important step necessary for bacterial access to the gut surface for adhesion, colonization, and persistence. As previously described, bacteria can initiate signaling pathways and following adhesion to host cells elicit host signaling cascades that are ultimately responsible for the final host phenotype (66, 132). Recognizing the likely evolutionary history that should exist between host and microbe in the case of *B. infantis* one would expect to find specific receptors that are the molecular binding partners of the bacteria. Consequently, one can expect that adhesion of *B. infantis* will initiate a cell-signaling cascade within the host.

The prevailing theory is that *B. infantis* competitively excludes other bacteria from adhering thus hindering pathogenic infections (89). While this certainly may play a role in the health benefits it is unlikely to be the complete story, and does not account for the claimed systemic health benefits that the microbes impart to the host.

Recent work by Desai *et al.* (32) demonstrated that food-borne pathogens and *B. infantis* bound various components of the ECM (31), suggesting that *B. infantis* may initiate signaling via ECM binding. Considering the evolutionary association and clinical demonstration of *B. infantis* with the host, it is logical that adhesion of *B. infantis* to the host cell invokes the host cell to change. It is very likely that this is done using known signal transduction routes that initiate new phenotypes to provide very specific benefits to the host. The lack of knowledge

surrounding the molecular mechanisms waits to be explored and defined. Creating an experimental model that allows direct, hypothesis-driven queries of the cell signaling pathways activated by *B. infantis* adhesion will provide great insight into the underlying mechanisms of improved host health could be uncovered. This would be a step to provide insight into host health as a whole, as well as a model for uncovering similar information about other microbial/gut interactions beyond those defined by infectious disease models.

CHAPTER III

MATERIALS AND METHODS

Mammalian Cell culture

Human colon colorectal adenocarcinoma Caco-2 cells (ATCC HTB-37) were grown in Dulbecco's modified Eagle's medium (DMEM) high glucose media with 2 mM L-glutamine and 1 mM sodium pyruvate, combined with %16.6 fetal bovine serum (FBS) by volume, 10 IU/ml Penicillin, 0.10 mg/ml Streptomycin and 1X non-essential amino acids (EmbryoMax ES Cell Qualified MEM, 100X, Millipore, Billerica, MA) in accordance with ATCC (American Type Culture Collection, Manassas, VA) recommendations. Due to the fact that these cells were to be co-cultured with bacterial cells an additional buffer composed of 10 mM 3-morpholinopropanesulfonic acid (MOPS), 15 mM HEPES, 10 mM N-(Tris(hydroxymethyl)methyl)-2-aminoethanesulfonic acid (TES), 2 mM sodium phosphate was added to further buffer the media (pH 7.2) (37). Incubation conditions were 37°C at 5% CO_2 in a humidified atmosphere. Cells were given fresh media according to need, but approximately every 2 d. Cells were grown in BD Falcon T-75 0.2 μm vented-cap, canted-neck flasks (Becton, Dickinson and Company, Franklin Lakes, NJ). During periods of extended culturing Caco-2 cells differentiate and exhibit a phenotype similar to that of intestinal epithelial cells. Time until differentiation is 16 d following confluence (126); to more closely mimic the conditions found in the human gut all adherence and gene expression assays will be done at day 21-22 post inoculation to allow for complete differentiation.

Final feedings 24 hr prior to the addition of bacteria were done with media lacking antibiotics and serum. This serum starvation was done to promote cell cycle synchronicity.

Bacterial Culture

B. infantis (ATCC 15697) was sub-cultured twice from freezer stocks to late stationary phase (36 hours) in deMan, Rogosa and Sharpe broth (MRS) containing an additional 0.5 % cysteine (Difco, Detroit, MI) at 37ºC using anaerobic conditions. The cells were centrifuged at a 5,500 X g for 1 min. to create a loose cell pellet after which the pellet was washed once with PBS and resuspended in mammalian cell culture media without serum (see above) at the required density of 10 X 10^6 CFU/ml (colony forming units). This cell suspension was used to interact with Caco-2 monolayers at a multiplicity of infection (MOI) of 1000:1 (1000 bacterial cells per Caco-2 cell) (120), which was approximately 10 ml of the afore mentioned cell suspension per T-75 flask.

Salmonella ser. Typhimurium LT2 (ATCC 700720) was grown, from freezer stocks, in Nutrient Broth (Difco, Detroit, MI) at 37ºC shaking at 220 rpm. After the first growth phase the cells were collected by centrifugation and transferred into Nutrient Broth for an additional 24 hr. The third transfer, used to perform the experiments in this study, was done in cell culture media lacking serum and antibiotics. After the third transfer, the organisms were grown for approximately 16 h (stationary phase). These cells were then collected by centrifugation and re-suspended in fresh cell culture media lacking serum and

antibiotics at the required density of 1 X 10^6 CFU/ml). These cells were subsequently used to conduct adhesion studies with Caco-2 cells at an MOI of 1000:1 (bacterial cell:host cell) in a final volume of 10 ml/flask.

Ganglioside Binding and Real Time-PCR

Gangliosides were purified from bovine buttermilk as described by Walsh and Nam (149). Briefly, the purification of gangliosides included ultrafiltration of fresh buttermilk (Gossner Foods, Logan, UT) with a 1 kDa membrane to remove the lactose followed by an organic extraction with chloroform:methanol:water (40:80:30). The purified gangliosides (6 µg) were used for immobilization with 100 g of 3 mm solid glass beads (Fisher Scientific, St. Louis, MO), which produced bioactive beads (gangliobeads) with a mixture of gangliosides on the surface as described by Walsh and Nam (149).

B. infantis was grown to exponential phase in media and conditions as described above. Bacterial cells were washed twice in saline and diluted to an OD_{600} of 0.2 in saline. This suspension (2 ml) was exposed to 10 beads for 10 min at 25˚C with agitation and washed three times with 50 mM Tris Cl (pH 7.2) for 5 min each. The presence or absence of *B. infantis* on the beads was quantified by RT-PCR (real-time-PCR) using universal bacterial 16S primers (IDT, Coralville, IA)

Following interaction with *B. infantis,* beads were washed in saline and added to 100 µl of distilled water and boiled 10 min to lyse bound bacterial cells. An aliquot of the boiled sample (12.5 µl) was used for RT-PCR with universal

bacterial 16S primers (IDT, Coralville, IA). Forward primer sequence was 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer sequence was 5'-ACGGCTACCTTGTTACGACTT-3'. The DyNAmo HS SYBR Green qPCR Kit (MJ Research, Waltham, MA) was used as per the manufacturer's recommendations in combination with a DNA Engine Opticon 2 thermal cycler (MJ Research). The thermal cycler was run for 50 cycles of 15 sec at 95˚C (melting), 30 sec at 60˚C (annealing) and 45 sec at 72˚C (extension and plate read). The threshold cycle number, C(t), was reported using the Opticon Monitor analysis software (Ver 2.02, MJ Research). Appropriate negative controls to probe for nonspecific binding (using 3 mm glass beads instead of gangliobeads) and sterility of the beads and buffers (using gangliobeads with saline instead of bacterial suspension) were also performed along with the test samples. The experiment was done in three biological replicates with RT-PCR in duplicates.

RT-PCR Data Analysis for Ganglioside Binding

A standard curve correlating log CFU with C(t) value was generated, by performing RT-PCR on samples of DNA extracted from log 5, 6,7 and 8 CFU/mL. Trend line analysis (performed in Microsoft Excel 2008 V12.0, Redland, WA) of the resulting C(t) values yielded the Equation (1):

$$C(t) = 2.624(\text{bacterial population}) - 40.77 \quad [r^2=0.97] \qquad \text{(Equation 1)}$$

C(t) values obtained from RT-PCR of the ganglioside-containing beads and glass beads and converted into bacterial population amounts using the equation generated from the standard curve. These values were then tested for

significance using an unpaired Student's t-test to compare the number of *B. infantis* adhered to the beads vs. the glass bead control. Statistical analysis was done using Prism 5.0 (GraphPad Software, San Diego, CA)

Host Gene Expression

Human Caco-2 cells were grown to confluence and allowed to differentiate following previously established protocols. Bacterial cultures were added at a MOI of 1000:1. At 30 min, 60 min and 120 min, respectively. Subsequently, 10 ml of Trizol LS (25), (Invitrogen, Carlsbad, CA) was added to the cell culture flask and incubated at 37°C for 15 min. Caco-2 cells were more thoroughly lysed by repeated pipetting of the Trizol LS followed by transfer to 15 ml conical and stored at -70°C until further RNA purification was done.

Trizol LS containing samples were freeze thawed three times (liquid N_2/60ºC) to further lyse host cells. The samples were then centrifuged at 8,000 rpm for 5 min to pellet cellular debris as well as bacteria that remain intact following Trizol LS treatment. The supernatant contained Caco-2 RNA. RNA isolation proceeded as per Trizol LS manufactures' protocol. RNA quantity was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc. Waltham, MA). Sample quality was assessed using the Bio-Rad Experion RNA StdSens analysis kit with their Experion automated electrophoresis station (Bio-Rad Laboratories, Hercules, CA).

Two biological replicate RNA samples were subjected to Affymetrix GeneChip One-Cycle Eukaryotic Target Labeling procedure according to

manufactures' recommendations. Briefly, sample RNA is reverse transcribed using T7 oligo (14) primer to create cDNA. This cDNA is again reverse transcribed using a T7 RNA polymerase, biotinylated nucleotide analogs and ribonucleotides to create labeled cRNA. These cRNAs were hybridized to a Human U133+2.0 Affymetrix GeneChip and further processed by the Genomics Core facilities at the Center for Integrated BioSystems Genomics Core (Utah State University). The arrays were processed using a GeneChip fluidics station 450, a GeneChip hybridization oven 640 and scanned with a GeneChip Scanner 3000 implementing Affymetrixs' GeneChip operating software (GCOS).

Statistical Analysis of Gene Expression Data

Using BioConductor (50) via a web based interface (http://cib-xcluster.biotec.usu.edu/BioC.html) the raw hybridization intensities from the arrays were pre-processed using the robust multi-array average (RMA) method (65). Following pre-processing the data were analyzed using the SAM (significance analysis of microarrays) plug-in for Microsoft Excel (144) to identify genes that were significantly differentially expressed with a false discovery rate (FDR) of less than 5%. The model used was a two-class unpaired time course, using the default settings. These significant genes were uploaded to Ingenuity Pathway Analysis (IPA) (116), (Ingenuity Systems Inc., Redwood City, CA) for pathway reconstruction and visualization.

Pathway Analysis Using Ingenuity Pathway Analysis

A core analysis using IPA (116) was done using the $\log_2$ ratios of treatments to control for the significant genes identified using SAM. Using the IPA library, each of the canonical signaling pathways was examined to determine what pathways contained genes from our dataset. The data set was overlaid onto the canonical pathways using the "overlay" feature. When protein complexes or groups of proteins were represented in a pathway, and found to contain a significantly changing gene, the "show members/membership" feature was used to identify what specific gene identified by the gene expression analysis was accounting for significance and what role that gene played in the complex or protein group. To create pathways for better visualization and for placement in the text of this document, relevant sections of canonical pathways were selected and edited in the "pathway designer" portal. All connections between proteins, small molecules, chemical reactions, etc., are linked to peer reviewed studies held in the Ingenuity database (use of this feature is available upon subscription only). Critical links relevant to this study have been cited in this work.

Gene Ontology Analysis

The list of significant genes ($\log_2$ ratio) were uploaded to GOEAST (157), a web based gene enrichment testing tool based on the gene ontology consortium (4) classification scheme, to determine what groups of molecular functions, biological processes or cellular components had higher than expected

significantly differentially expressed genes. Default settings were used with the exception of the significant level of enrichment, which was set at q≤ 0.01.

Protein Sample Collection

Co-cultures from which proteins were sampled were created in the exact same conditions as previously described for the gene expression samples through the incubation periods with the bacteria. Following interaction with the respective bacterial treatment, media in flasks was removed and cell layers were washed with PBS to remove non-adherent bacteria and cellular debris. The co-cultures were scraped from the flask and re-suspended in 1 mL cell lysis cocktail modified from Pawson *et al*. (109) in 30 mM HEPES (pH 7.4) containing 1 mM EDTA, 50 mM Sodium pyrophosphate, 100 mM sodium fluoride, 10 mM orthovanadate, and Roche protease inhibitor cocktail tablet (added fresh, per manufactures recommendations - 1 tablet per 50mL buffer) (Roche Indianapolis, IN). The cell suspension and lysis buffer were then added to 2 mL screw cap tubes with 0.5 g of 0.1 mm glass beads and bead beat in a BioSpec mini-bead beater (BioSpec Products, Bartlesville, OK) for 30 seconds. The samples were stored at -80°C for further analysis.

Protein Concentration Determination

Protein concentrations were determined using the Bio-Rad *DC* Protein Assay kit according to the manufactures' protocol, with the exception of using Bovine Serum Albumin (BSA; catalog number 15561-020) from Invitrogen in

place of the provided protein standard. The assay was performed in a Nunc F96 MicroWell plate using a Molecular Devices SpectraMax Plus 384 plate reader. Analysis of the readings was done using Molecular Devices SoftMax Pro software (version 3.1.2). All standard and sample concentrations were quantified in duplicate.

Cytotoxicity Assay

To assess the impact of the presence of *B. infantis* to the viability of the host model, a cytotoxicity assay was performed. Caco-2 cells and bacterial cells were all cultured as previously described, except that Caco-2 cells were cultured in 12-well plates instead of flasks. Bacteria were added to the differentiated Caco-2 monolayers according to the scheme presented in Fig. 4. Following the 120 min. incubation, monolayers with microbes were washed with PBS to remove non-adherent bacteria, and treated with 1X porcine trypsin for 5-7 min. Adding fresh cell culture media containing serum and buffer without antibiotics stopped trypsinization. This suspension was divided in half, and used for analysis of total cell count and viable cell count using a NucleoCounter (ChemoMetec A/S Denmark) according to the manufacture recommendations. Briefly, for total cell counts (TC), 100 μl of lysis buffer followed by 100 μl of fixing buffer to 100 μl of cell suspensions and briefly mixed using a vortex. For suspensions to assay viable cell count (VC) 100 μl of suspension was combined with 200 μl PBS and mixed. Each treatment was measured in using two biological replications.

Statistical Analysis of Cytotoxicity Assay Data

Cell counts obtained in the cytotoxicity assay were converted into percent survival using Equation 2:

$$\text{Percent viable cells} = ((TC-VC)/(TC))*100 \qquad \text{(Equation 2)}$$

TC = total cell count, VC = viable cell count. Statistical analysis was done using JMP V7.0 (SAS Institute Inc., Cary, NC). A Tukey's *post hoc* analysis was done to determine the statistical differences between the levels within a treatment.

Western Blot Analysis

The sample loading dye and preparation buffer consisted of 37.5 mM Tris (pH 6.8) containing 137.5 mM glycerol, 73.75 mM SDS, 1.15 M β-mercaptoethanol to reduce disulfide bonds in the proteins, and a few grains of brilliant blue dye to act as a marker of sample migration. Protein samples at a concentration of 50 mg/mL were added to the sample dye and preparation buffer and denatured by boiling for 5 min. Protein samples were then loaded in 10% Tris-HCl (pH 6.8) polyacrylimide gels and run in the Bio-Rad Mini-PROTEAN 3 cell using a buffer containing 25 mM Tris base (pH 10.7), 192 mM glycine and 3.5 mM SDS (pH 8.3) at 110 V until the dye front had reached the bottom of the gel. The standard used was MagicMark XP western protein standard from Invitrogen. The separated proteins were transferred to a PVDF membrane (Thermo Scientific product number 88518) using a transfer buffer consisting of 25

mM Tris base (pH 10.7) containing 200 mM glycine and 20% (v/v) methanol in water. The transfer was done using 100 V for 70 min. After transfer the blot was probed for the presence of specific proteins corresponding to the potential signaling pathway determined using the gene expression data using commercial antibodies (TABLE 2). Western blots were done using the Pierce Fast Western Blot Kit, according to the manufactures recommendations, with an additional 60 min blocking step with 2% non-fat dry milk in 20 mM Tris base (pH 10.7), 0.5 M NaCl (TBS), immediately before adding the primary antibody solution.

If cross-reactivity was present that interfered with band development using the rapid method the analysis was repeated using a classical method (137). The classical method used a membrane blocked with 2% non-fat dry milk (Wal-mart Stores Inc., Bentonville AR) in TBS buffer (pH 10.7) for 60 min. The blocking buffer was removed and the blot was washed with the primary antibody overnight at 4ºC. The primary antibody was re-suspended in a buffer containing 2% non-fat dry milk, 20 mM Tris base (pH 10.7), 0.5 M NaCl and 1 mM Tween 20 (TTBS buffer). Following overnight mixing the blot was washed 2X for 10 min with TTBS (pH 10.7) buffer without non-fat dry milk and the washes were discarded. The blot was incubated at room temperature with the secondary antibody solution for 120 min. The secondary antibody solution contained suspended a secondary antibody, conjugated to horseradish peroxidase, in TTBS buffer (pH 10.7) with 2% non-fat dry milk.

TABLE 2. Primary antibodies used in this study. All antibodies were purchased from Novus Biologicals (Littleton, CO) except β-actin, which was purchased from Sigma-Aldrich (St. Louis, MO). Antibodies targeted toward specific phosphorylation states of the proteins that correlate to active signaling states are designated as phospho- and the target's known phosphorylation site.

Antigen	Species and Type	Dilution or concentration used
GPR20	Rabbit Polyclonal	2 µg/ml
GPR161	Rabbit Polyclonal	2 µg/ml
PYK2	Rabbit Polyclonal	1:500
PYK2 phospho-Tyr402	Rabbit Polyclonal	1:1000
PDK1	Rabbit Polyclonal	2 µg/ml
PDK1 phospho-Ser241	Rabbit Polyclonal	2 µg/ml
AKT1	Rabbit Polyclonal	1:2000
AKT1 phospho-Thr308	Rabbit Monoclonal	1:10000
AKT1 phospho-Ser473	Rabbit Monoclonal	1:10000
TNFAIP3 (A20)	Mouse Monoclonal	2 µg/ml
BIRC3 (aka cIAP2)	Rabbit Polyclonal	1:2000
SERPINB9	Mouse Polyclonal	1:500
β-actin	Mouse Monoclonal	1:10,000

Following mixing the antibody solution was discarded and the blot was washed 5X for 10 min. in TTBS (pH 10.7) without non-fat dry milk. The blot was protein side up on plastic wrap, exposed to the western blotting detection reagent (Amersham ECL Plus) so as to cover the membrane completely, and incubated for 1 min. Subsequently, the blots were imaged using the chemiluminescent detection with a FujiFilm LAS-3000 imaging system V 2.0 (FujiFilm U.S.A. Inc, Valhalla, NY) on the highest setting for 5 min. Image files were analyzed using FujiFilm Multi Gauge software to detect the bands. To validate constant sample concentration, blots were stripped and re-probed for β-actin (26). Stripping was done in a solution containing 62.5 mM Tris base (pH 6.8) containing 69.5 mM SDS and 100 mM β-Mercaptoethanol, at 50ºC for 20 min. Stripped membranes were briefly washed in TBS (pH 10.7) without non-fat dry milk followed by sterile water for 5 min. The membranes were probed as previously described using the Pierce Fast Western protocol with the previously described additional blocking step.

Adhesion Assay

Caco-2 cells were cultured as described above and plated at a density of 10^5 / cm^2 in a 96 well plate. Following differentiation, media from the caco-2 cells was removed and the cells were incubated for one hour at 37°C (5% CO_2) in 50 µl of GPR161 or GPR20 antibody serially diluted in DMEM. After 1 h, 50 µl of bacterial suspension washed in PBS (pH 7.4) and resuspended in the DMEM at 10^8 CFU/ml was added to the caco-2 cells for an MOI of 100:1 and incubated for

an additional hour. The non-adherent bacteria were removed via aspiration and the caco-2 cells were washed three times with 200 µl of 1X Tyrode's buffer (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, 10 mM sodium pyruvate (pH 7.4)). The caco-2 cells were exposed to 50 µl of DNA extraction buffer (AEX Chemunex, France). The cells were incubated at 37°C for 15 minutes and then at 95°C for 15 min. The cell lysates were stored at -70°C.

Quantitative PCR (qPCR) was used to quantify the number of adherent microbes to caco-2 cells. Primer pairs for the desired microbes and caco-2 monolayer were designed using Primer-BLAST (125). Primers used to quantify *B. infantis* amplified the fructose-6-phosphate phosphoketolase gene (BLON_1722) (Forward-GAC AGA GCG TAA CCC AGC TC, Reverse- ACT ACC CCT GGC CTG AAC TT). Primers used to quantify Caco-2 cells amplified G3PDH gene (Forward-ACC ACA GTC CAT GCC ATC AC, Reverse- TCC ACC ACC CTG TTG CTG TA). qPCR was done using iQ SYBR Green qPCR mix (Bio rad, Hercules, CA) as per the manufacturer's recommendations using a CFX-96 Real-Time PCR detection system (Bio rad, Hercules, CA). Standard curves with known number of bacteria (determined by plate count) and know number of Caco-2 cells (determined by direct microscopic count) were generated (data not shown). Based on the standard curve the number of bacteria present per caco-2 cell were determined. The results were plotted and error bars added to the plots

using the standard error of the mean of the 4 observations made per treatment class.

CHAPTER IV

RESULTS

While the current field of host/microbe relationships is quickly expanding, little is known about host/probiotic microbe relationship. At the same time use of probiotic bacteria in food products is increasing with increasingly broad and undefined health claims, most of which remain to be substantiated. In consideration of the use of B. infantis and it's role in the infant gut this study was undertaken to determine the biological importance, molecular mechanisms and resulting cellular phenotype induced *in vitro* by the interaction between an human intestinal epithelial model and *B. infantis*. Using a systems biology approach, the resulting host reaction to B. infantis was characterized as well as the signals responsible for producing the host phenotype to test the hypothesis that B. infantis binds host GPCRs to induce new and beneficial activities.

Epithelial Cytotoxicity

The cytotoxicity of gut epithelial cells was assayed to determine if host cell survival was impacted by adhesion of bacterial cells. Adhesion of *B. infantis in vitro* to gut epithelial cells significantly ($p=0.006$) increased the viable cell population. Unfortunately, addition of B. infantis simultaneously with *Salmonella* ser. Typhimurium did not rescue the host cell from death (Fig. 5). While *B. infantis* did not rescue *Salmonella* ser. Typhimurium–induced cytotoxicity, it did increase epithelial survival when presented alone. Consequently, the

remaining experiments focused on the interaction between the host and *B. infantis* to define mechanisms that increased survival of gut epithelial cells.

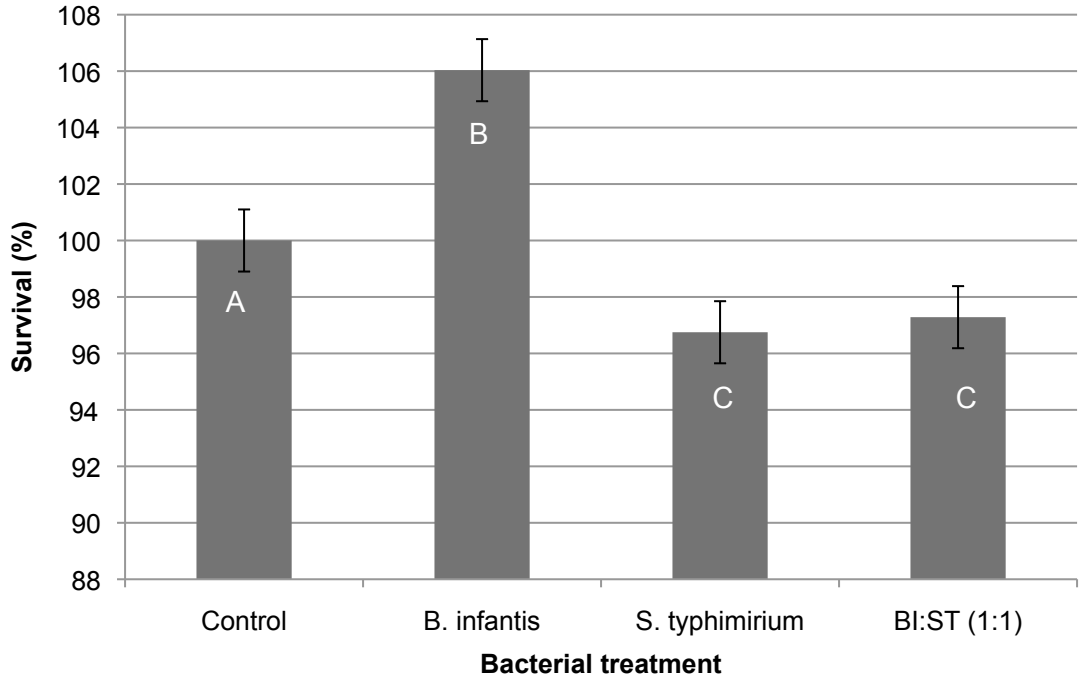

FIG. 5. Gut epithelial cytotoxicity with addition of *B. infantis* (BI) and *Salmonella* er. Typhimurium (ST) after 60 min of co-incubation. The organisms were added at equal concentrations to the cell culture (1:1). Bars with the different letters indicate significantly difference responses ($p<0.05$).

Considering the decreased cytotoxicity imparted by B. infantis, the question becomes how does it impart this effect since this organism is not invasive, but does adhere to the epithelium? No receptors for *B. infantis* adhesion to host cells are known. Consequently, further experiments tested the

hypothesis that *B. infantis* bound specific host protein receptors that directly transduced intracellular signal transduction pathways to increase cell survival. Without knowledge of a specific receptor we used gene expression regulation during *B. infantis* adhesion to uncover the underlying molecular changes in the membrane proteins that are known to trigger or modify the cell cycle and survival.

To begin glycolipid adhesion was done based on the observations of Desai et al. (31), to establish a link between gangliosides and cell signaling as others have demonstrated with other microbes (3, 97, 99). Significantly ($p<0.001$) more *B. infantis* cells were bound to the beads by 10-fold as compared to the control. (Fig. 6A). Gangliosides are commonly used by viruses and bacteria to gain entry into the host cell (141, 142). Consequently, *B. infantis* was added to the gut epithelia to demonstrate that the microbes also bound GM1 (Fig. 6B) to determine if this interaction is relevant in vitro. Blocking the GM1 receptor on caco-2 cells significantly ($p=0.05$) reduced *B. infantis* adhesion by ~10%. Together, these observations indicate that this probiotic microbe uses gangliosides during adherance to gut cells. It is unlikely that this is the only receptor used. Subsequently, gene expression was used to discover additional receptor proteins that may be used by B. infantis to induce signal transduction pathways that are linked to decreased cytotoxicity.

Gangliosides are directly linked to host signal transduction through GPCRs (55, 97, 99).

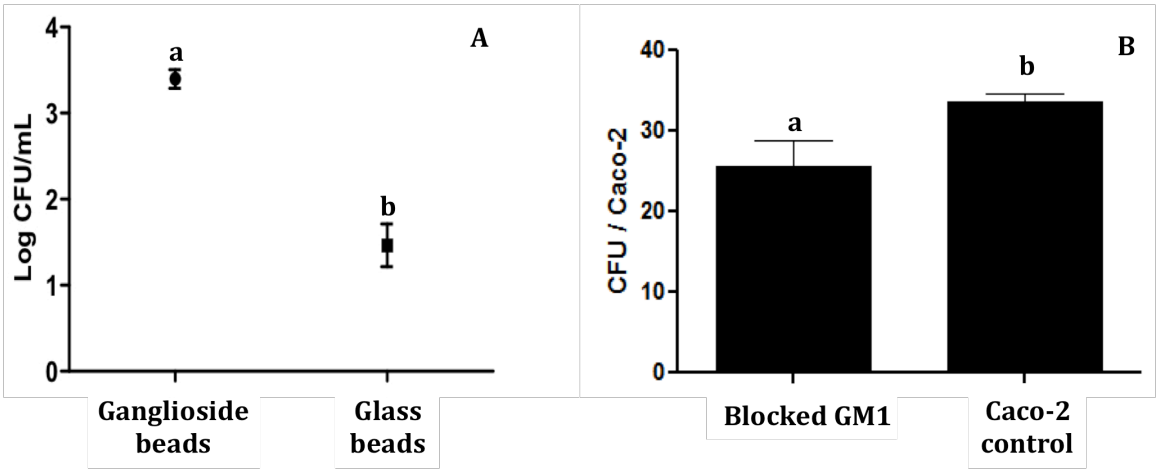

FIG. 6. *B. infantis* binding of mixed gangliosides on glass beads. (panel A) and to caco-2 cells with before and after blocking GM1 with antibody (panel B). In each panel the treatments containing different letters indicate significant differences between the treatment and control ($p \leq 0.05$).

The observations of *B. infantis* increasing cell longevity and binding gangliosides suggests that signaling is initiated following adhesion that results in increased cell survival initiated via GPRC signaling. Therefore, the hypothesis that *B. infantis* interacts with host GPCRs during adhesion to decrease cytotoxicity was tested.

Gene Expression Changes During *B. infantis* Adhesion

Adhesion of *B. infantis* to intestinal epithelial cells initiated significant ($q<0.05$) regulation of 208 genes in caco-2 cells over 120 minutes co-incubation, which is ~0.4% of the entire the host genome (TABLE A.1). Gene regulation was almost entirely induction that increased over incubation time, indicating that the longer association of this non-invasive microbe initiated gene expression

changes that led to increasingly pervasive changes in the host cell. The phenotypic changes observed (Fig. 1) occurred within 120 min; therefore, the set of genes differentially expressed within the 210 genes mediated increased survival. *B. infantis* does not invade the host (103). Consequently, cellular changes must be transduced via membrane receptors during *B. infantis* adhesion.

Considering that the set of induced genes were involved in very diverse cellular processes and functions, gene ontology (4) (GO) enrichment analysis (21) was done to highlight the functional associations based on the significantly differentially expressed genes and reduced cytotoxicity. Significantly enriched categories were found in each of the three functional GO categories (TABLE 3). Significant GO nodes contained between 2 and 70 genes within each node. Based on the specific relationship to cell death, categories were selected for further investigation to define the molecular mechanism(s) that increased cell survival (Figs. B.1-B.3).

Critical examination of the GO enrichment analysis to specifically examined categories associated with decreased host cytotoxicity were identified . The only significant GO term in cellular component category was “nucleus,” GO:004066 ($p=3.24 \times 10^{-5}$), representing the gene expression regulation via new transcription factors. Additional examination of genes from this category (Fig. 7) showed that genes were induced at 60 min. and they remained induced at 120 min. This temporal pattern was observed in other GO categories as well.

TABLE 3. Significantly enriched GO categories during *B. infantis* association with intestinal epithelial cells in vitro. Some genes occur in multiple nodes, so the total number of genes in the analysis do not sum to 210.

GO Category	GO Term	Annotation	Number of molecules in category out of 210	p-value
Cellular Component	0043066	nucleus	70	3.24×10^{-05}
Biological Process	0006350	transcription	42	6.39×10^{-6}
	0006915	apoptosis	15	1.90×10^{-4}
	0030198	extracellular matrix organization	5	1.67×10^{-4}
	0043066	negative regulation of apoptosis	12	1.11×10^{-5}
	0043124	negative regulation of I-kappaB kinase/NF-kappaB	2	4.14×10^{-4}
	0042994	cytoplasmic sequestering of transcription factors	2	2.26×10^{-4}
	0006355	regulation of transcription, DNA-dependent	46	3.10×10^{-6}
	0060056	mammary gland involution	3	7.93×10^{-8}
	0001843	neural tube closure	3	1.23×10^{-4}
	0006954	inflammatory response	10	1.23×10^{-4}
	0006935	chemotaxis	7	1.75×10^{-4}
	0007567	parturition	2	5.19×10^{-4}
Molecular Function	0003700	transcription factor activity	33	5.29×10^{-10}
	0008140	cAMP response element binding protein binding	3	2.60×10^{-6}
	0008009	chemokine activity	4	1.45×10^{-4}
	0005523	tropomyosin binding	3	1.23×10^{-4}
	0017022	myosin binding	3	3.86×10^{-4}
	0004887	thyroid hormone receptor activity	2	8.82×10^{-4}
	0043565	sequence specific DNA binding	20	2.78×10^{-7}

Additionally, significantly enriched molecular functions were “transcription factor activity” GO:0003700 ($p=5.29 \times 10^{-10}$) and “sequence specific DNA-binding” GO:0043565 ($p=2.78 \times 10^{-7}$). Further examination of these categories provided a list of over enriched transcription factors responsible for the transcription of the genes involved in the host response (Fig. 8-9). Finally, the biological process GO category contained significantly enriched genes in “negative regulation of apoptosis” (GO:0043066) ($p=1.1 \times 10^{-5}$), with 12 of the 210 (5.7%) of the total significantly regulated genes in this node. Examination of this category found genes induced with increasing time at 60 min and 120 min adhesion of *B. infantis* that are directly related to the observed phenotype of the epithelial cell (Fig. 10). As such, mechanisms of the enriched apoptotic genes were visualized using signal transduction pathways to discern possible routes that explain the phenotype.

To determine which network and signal cascade molecules were related, the list of significantly regulated genes analyzed as a set of pathways that mediate apoptosis with particular attention to networks related to GPCRs and gangliosides. In total, these analyses pointed to a set of genes associated with GPCRs and AKT signaling, which is a central regulatory molecule in many signal transduction pathways, including cell survival, and associated specific signaling molecules with cell surface receptors (Fig. 11). Overlaying the host gene expression data during *B. infantis* association found GPCRs and NF-κB to be regulated.

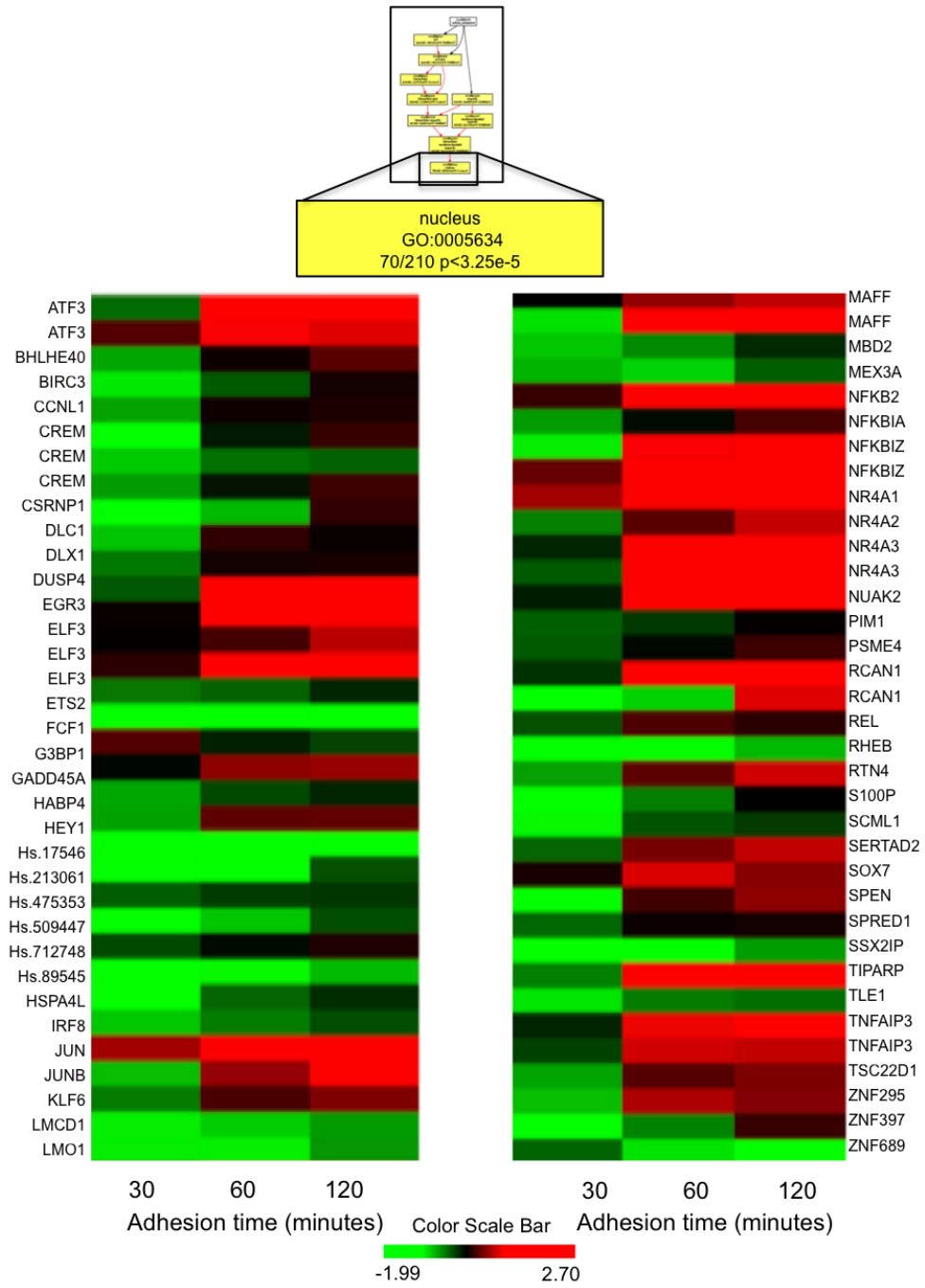

FIG. 7. GO Tree and Heat Map of Cellular Component; Nucleus during co-culture incubation. Multiple genes are listed as the gene chip contains multiple probesets for the same gene. The complete GO tree for cellular component is presented in Fig B.3.

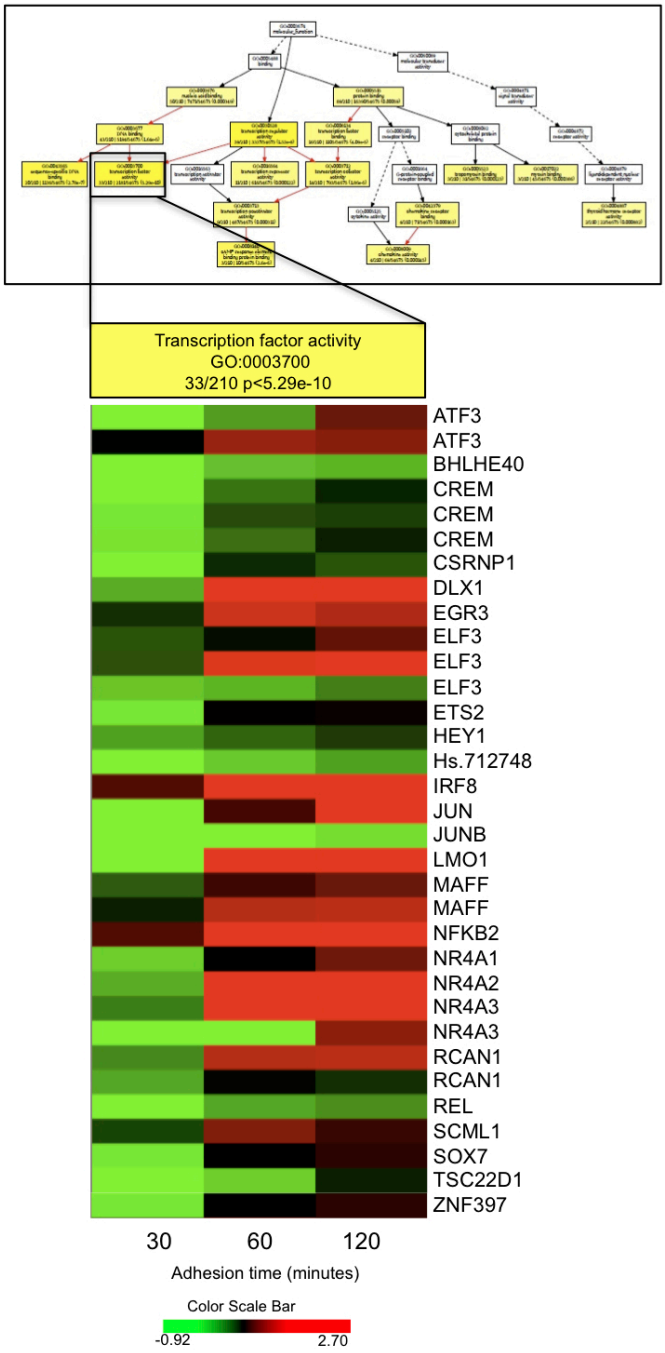

FIG. 8 GO tree and heat map of the Molecular Function category Transcription Factor Activity during co-culture incubation. Multiple genes are listed as the gene chip contains multiple probe sets for the same gene. The complete GO tree for molecular function is presented in Fig. B.1

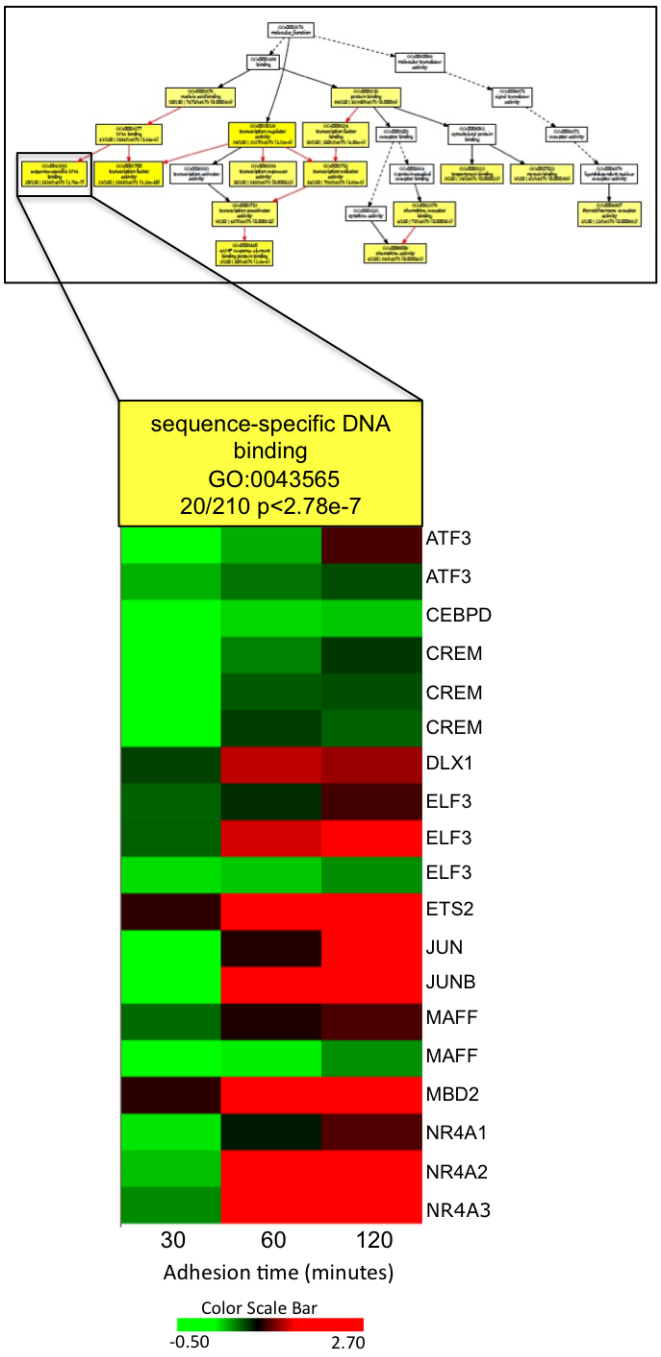

FIG. 9. GO tree and heat map of molecular function for sequence specific DNA-Binding activity during co-culture incubation. Multiple genes are listed as the gene chip contains multiple probe sets for the same gene. The complete GO tree for molecular function is presented in Fig. B.1.

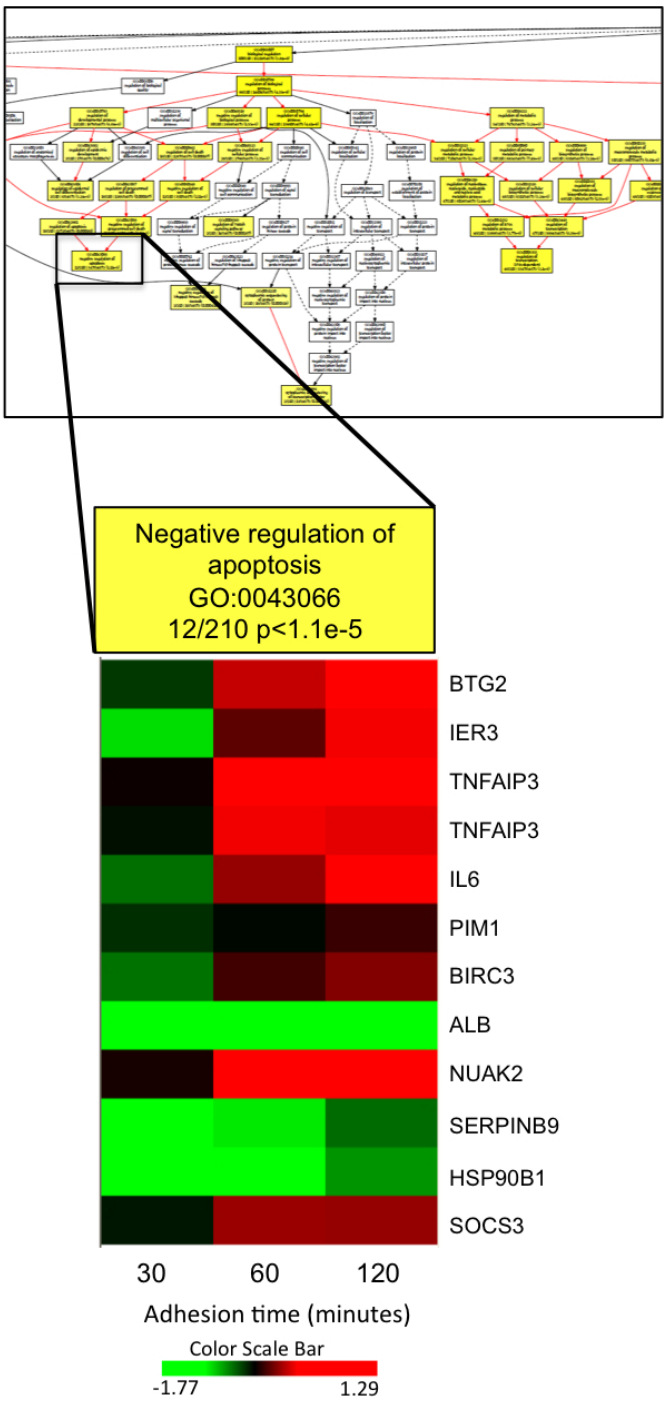

FIG. 10. GO tree and heat map of the biological process Negative Regulation of Apoptosis during co-culture incubation. The complete GO tree for biological process is presented in Fig. B.2.

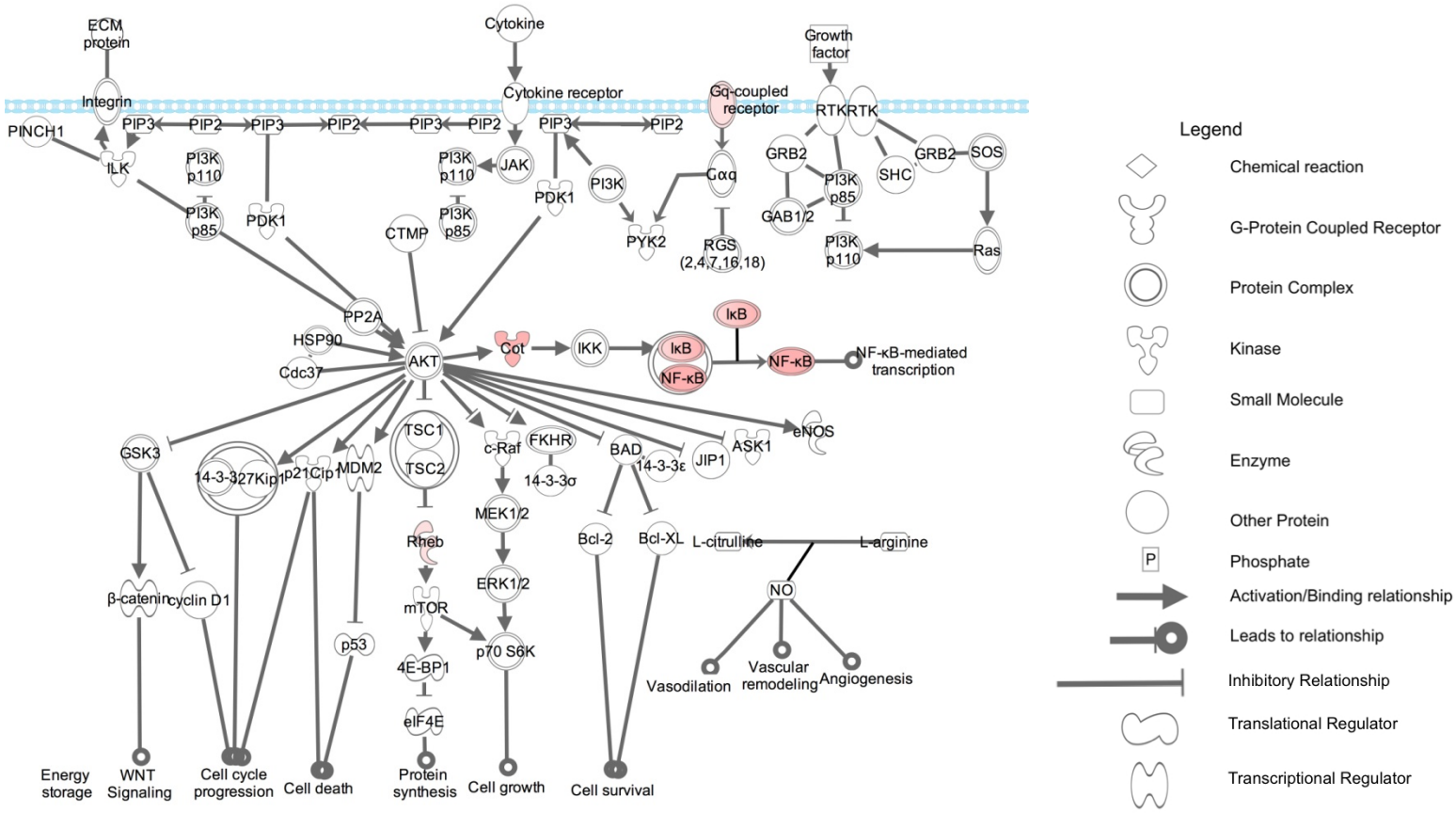

FIG. 11. Potential surface receptors and signaling pathways. Red fill indicates significant ($q<0.05$) induction of gene expression resulting from *B. infantis* association. Shading of the fill indicates the intensity of increase in regulation.

However, AKT gene expression was unchanged. NF-κB is a transcription factor and was implicated as involved in the transcription of the negative regulation of apoptosis genes during GO enrichment (Fig. 11).

Considering the direct signal transduction link between AKT and NF-κB a more focused analysis was done to more clearly define the specific interactions with membrane initiated induction as the beginning point (Fig. 12). Two GPCRs were induced, and tied with the increased expression of three genes GO analysis associated with the negative regulation of apoptosis. The induction of membrane bound cell signaling receptors by the presences of *B. infantis* lead to the hypothesis that GPR20 and GPR161 were the receptors bound by *B. infantis* adhesion to initiate AKT-mediated signaling to reduce cytotoxicity. To determine if these proteins were involved in host-microbe binding, an adhesion assay was done after blocking GPR161 and GPR20 with specific antibodies. Blocking GPR161 and GPR20 did not significantly ($p>0.05$) change the number of adherent bacteria adhered to the cell membrane, indicating that these two proteins are not acting as the binding site for *B. infantis* (Fig. 13). These observations eliminated GPR20 and GPR161 as adhesion partners for *B. infantis*, their role in the host-microbe relationship is still unknown, as are their potential to activate signaling cascades in the host cell. While GM1 was a receptor for B. infantis, the protein interaction remained unclear. Consequently, confirmation of AKT was pursued by measuring the protein level and activation state (i.e. phosphorylation) of specific signaling molecules up and down stream of

AKT were done to define the signaling network between the membrane and NF-κB.

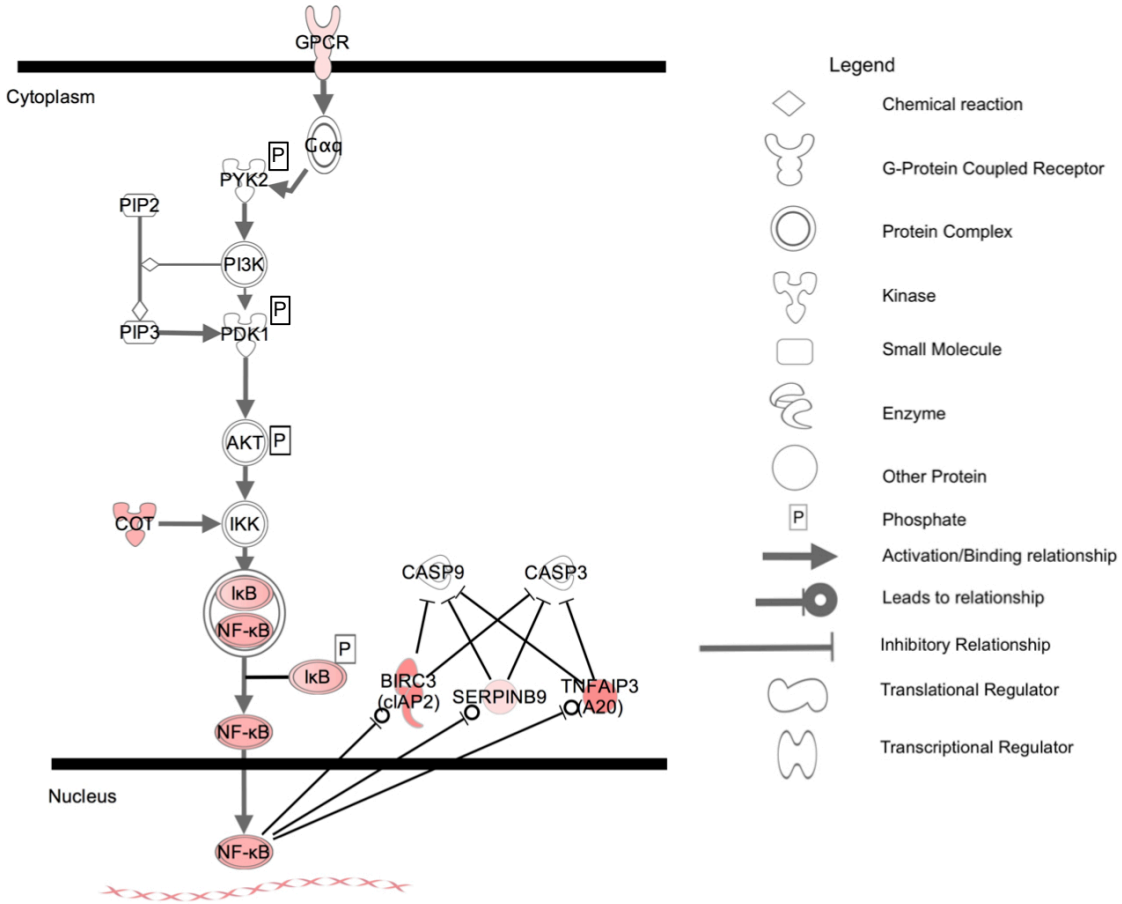

FIG. 12. G-protein coupled receptor signaling induced by *B. infantis* adhesion. Red fill shading indicates the intensity of gene expression induction during *B. infantis* in vitro association with gut epithelium. Red fill indicates significant ($q<0.05$) induction of gene expression resulting from *B. infantis* association. Shading of the fill indicates the intensity of increase in regulation.

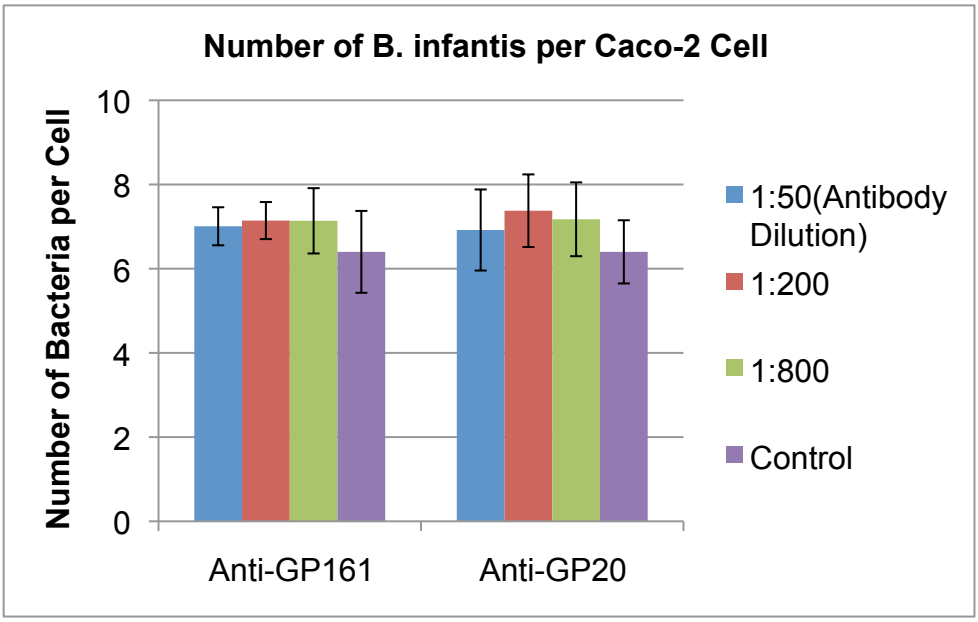

Fig. 13. Adhesion assay. Blocking of GPR161 and GPR20 did not decrease *B. infantis* binding, demonstrating they are not the receptors for adhesion.

Despite the unknown role of GPR20 and GPR161 (58, 96) and being ruled out as a receptor for *B. infantis* confirmation of the protein change was evaluated. The amount of GPR20 protein did not significantly change with *B. infantis* treatment over time. However, GPR161 protein content did increase with treatment and increased slightly over adhesion time (Fig. 14).

Transduction between GPCRs occurs via an adaptor protein (Gαq) with PYK2. The protein level and phosphorylation status remained constant between treatments and with time of PYK2. Since the protein was constitutively phosphorylated, it suggests that GPCRs were constantly triggering activation due

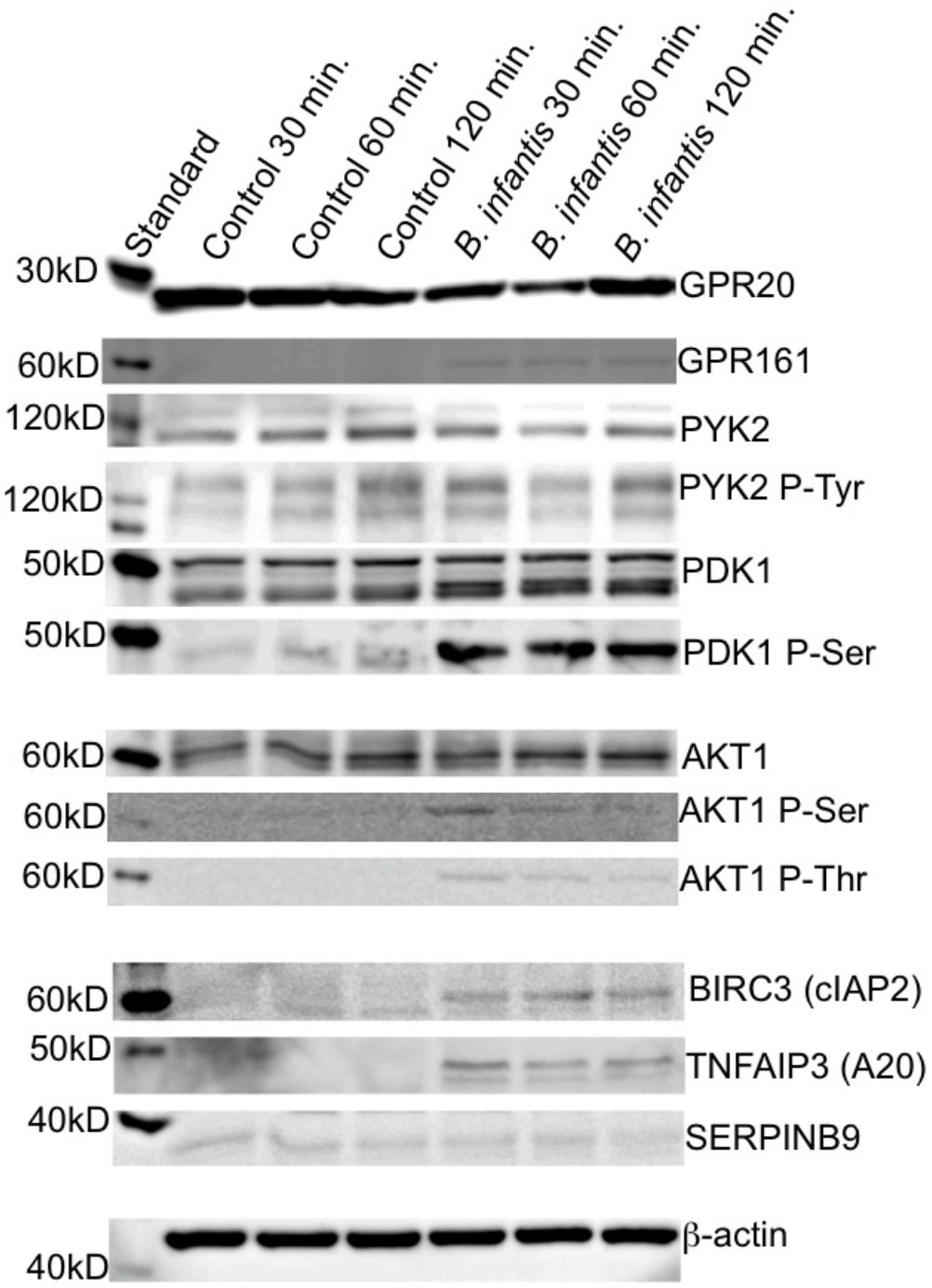

FIG. 14. Images of western blots probing signaling cascade. Protein samples used in this experiment were total protein samples.

to established link between GPCRs to PYK2 (34). Unfortunately, the exact remains to be defined. However, it does indicate that membrane mediation via PYK2 is involved in *B. infantis* adhesion. Subsequently, examination of the protein level and phosphorylation status of PDK1, the next protein involved in AKT-mediate signaling was examined. The protein level of PDK1 remained constant during time and *B. infantis* treatment, as indicated with gene expression. However, phosphorylation of PDK1 at Ser241 increased with addition of *B. infantis*, suggesting that activation of PDK1 kinase activity (18) and subsequent phosphorylation of AKT (8, 100).

AKT, PKY2, and PDK1 are activated by phosphorylation, which enables the signaling cascade to progress (48, 130, 134). AKT1 protein concentration remained constant in the control and *B. infantis* treated cells. The host had a small amount of phosphorylation at Ser473 that decreased with time. In contrast, the phosphorylation status, and thereby activation status, of AKT increased at Ser473 and Thr308 with the addition of *B. infantis* (Fig. 14). Phosphorylation at both sites decreased with time. Thr308 phosphorylation of AKT was only observed in the *B. infantis* treated cells indicating that the bacterial adhesion led to full and complete activation of AKT. Without phosphorylation at Thr308 AKT activity is greatly reduced nearly to the point of loss of function, and as such phosphorylation at Thr308 is considered a master regulator for AKT activity (100).

The gene expression results indicated induction of BIRC3 (cIAP2), TNFAIP3 (A20) and SERPINB9. SERPINB9 protein levels were not affected by *B. infantis* adhesion (Fig. 14), while both BIRC3 (cIAP2) and TNFAIP3 (A20) showed increased protein levels in the *B. infantis* treated cells at all time points.

CHAPTER IV

DISCUSSION

Adhesion of *B. infantis* to a host gut epithelial model significantly increased the survivability of the host cells. Although when *B. infantis* was mixed with *Salmonella enterica* ser. Typhimurium and added to the host cells a similar increase in cell survival was not observed. This indicates that *B. infantis* may protect host cells rather than rescue them from some challenge. Similar observations have been made with the probiotic *Lactobacillus rhamnosus*, another purported probiotic, when caco2 cells were treated with the probiotic and then challenged with *Salmonella* ser. Typhimurium (13). The authors did not show data or discuss if the host cells treated with *L. rhamnosus* showed decreased cytotoxicity, however. Contrary to these findings, Lee Do *et al.* (84) found that *Bifidobacterium adolescentis*, another probiotic microbe, generated peptidoglycan structures that inhibited caco2 cell growth in a dose dependent manner, while yet another study with *B. adolescentis* showed no increase influence to cell survivability in bacterial treated cells as compared to the control (45). Literature reports of specific molecular explanations of probiotic/host interactions contain conflicting and confusing reports. Consequently, this study sought to provide orthogonal methods of confirmation to prove specific molecular interactions and signaling routes.

Results from this study demonstrated an increased cell survival. Considering that contradictory results between *B. infantis* and *B. adolescentis*

treatment, it is likely that this trait is strain dependent. This strain difference points out the desperate need for genome sequencing and genetically defined models for understanding the molecular mechanisms underlying the complex host-microbe relationship as was done in this study. With the disparity of cytotoxic effects of other probiotics, it can reasonably be concluded that the increased host cell survival that is observed in *B. infantis* treated cells may not be novel, but certainly is significant as it has not been observed with other probiotics. Increased cell survival could be the underlying mechanism to the overall improvement in host health observed in humans harboring this probiotic.

It was found that *B. infantis* bound gangliosides. Gangliosides are glycolipids with a complex set of structures involved in lipid rafts (3) and are linked to neurological function. The terminal residue is often sialic acid (147). During viral and bacterial adhesion they are commonly used as a non-specific binding partner that enables close association that allows subsequent specific protein association for invasion (141, 142). They are also directly linked with triggering cellular signaling cascades (55, 97, 99). This result further encourages investigation of the host gene expression.

GO enrichment analysis of gene expression data generated from host cells treated with *B. infantis* revealed the biological process “negative regulation of apoptosis” to be significantly over-represented. Molecular function and cellular component GO results indicated that large transcriptional changes occurred as a result of *B. infantis* adhesion.

Numerous other studies discussed the impact probiotics have on intestinal epithelial cell health and, attribute those effects to prevention of pathogenic adhesion, improved cell barrier function, absorption and metabolism of bacterial toxins, and the production of anti-microbial peptides (6, 12). However, these studies do not show that probiotics can actually increase cell survival as was indicated by this gene expression study and cytotoxicity assay.

Western blot analysis of the proteins implicated by the gene expression and GO enrichments to play a role in altering host cell survival revealed a likely mechanism by which the increase in survival was observed. Perhaps the most significant observation from this work is the complete activation of AKT. Substrate binding and increased rate of enymatic atalysis by AKT requires both Ser473 and Thr308 phosphorylation to occur with phosphorylation at Ser473 often preceding and usually being a necessary pre-condition of phosphorylation at Thr308 (100, 130). Only in the *B. infantis* treated cells are both of the activation sites for AKT seen as phosphorylated leading to complete activation of the kinase.

Pathway analysis showed that AKT activation is related to the translation of three proteins with ties to increased cell survival (10, 62, 138, 139). Three proteins - cIAP2 (BIRC3), TNFAIP3 (A20) and SERPINB9 - were all induced in *B. infantis* treated cells, and not in the other cell treatments, with the exception being SERPINB9 as it was found in the control cells. These proteins are downstream of NF-KB and known inhibitors of caspases that are needed for

apoptosis (28, 33). Induction of these genes and proteins to block caspase activity is one explanation of increased cell survival with addition of *B. infantis*.

Other studies of probiotic interactions with host cells have queried AKT activation as well, but did not query AKT for complete activation. Yan *et al*. (155) showed that *L. rhamnosus*-treated cells led to the phosphorylation of AKT at Ser473. It was previously mentioned that for complete activation of AKT and subsequent signaling activity, AKT must be phosphorylated at both Ser473 and Thr308. Without being completely phosphorylated the ability of AKT to activate NF-KB and initiate NF-KB's subsequent effect on host transcription is greatly reduced. Yan et al. did not test this and so conclusions about whether or not *L. rhamnosus* can fully activate AKT cannot be reached.

In light of the data from this work it is likely that activation of AKT and the subsequent translation of cell survival proteins would be observed in other probiotic systems. As previously mentioned, other studies attribute the increase in cell survival observed in probiotic treated cells to some of the other actions probiotics are observed to render to the host. No other study specifically postulated that the actual adherence of the probiotic to the host cell set off signaling cascades increasing host cell survival as has been demonstrated in this study.

The observed increase in protein concentration of proteins with known ties to increased cell survival a more complete picture of improved host health can be formed. In addition to likely excluding pathogenic bacteria from gaining access to

host cells, *B. infantis* adhesion increased the host cell survival rate, as well as responsible for producing anti-microbial compounds known to kill pathogens. The sum total of the production of anti-microbial compounds, physically excluding pathogenic bacteria, increasing production of cell survival proteins and suppression of inflammation is most likely results in the improved health observed in clinical trials and animal studies.

The implications of the identification of two GPCRs identified as involved in the signaling resulting in the increased cell survival of the host are unclear. Gene expression analysis shows these two receptors as potentially involved, while western blot analysis confirms the presence of these two receptors. GPR161 and GPR20 are both non-sensory, orphan GPCRs (58, 96). Orphan receptors are categorized as such because they do not have any known ligands (96). Very little work has been published on these two receptors. This study is the first to identify two GPCRs as potentially involved in the manifestation of the health benefits of probiotics.

Bioinformatic examination of the two receptors' protein sequences using a transmembrane region predictor (60, 101, 102) and an N-glycosylation predictor (114) reveals two potential sites for N-linked glycosylation to occur (Figs. C.1-C.2). N-linked glycans are post-translational modifications that result in the covalent bonding of complex oligosaccharides to asparagine residues in proteins. These glycans have a basic core structure Man3GlcNAc2Asn (147), followed by various other terminal sugars, including sialic acid. As previously discussed,

these carbohydrates are excellent binding partners for bacteria (11). Gangliosides are also often terminally modified with sialic acids (147), which have already been shown to be bound by *B. infantis*. The fact that both of these glycans share terminal sialic acids as a characteristic could provide a link between ganglioside binding and the implied involvement of these GPCRs in the adhesion of *B. infants* and subsequent cell signaling events that followed. The relationship between glycans, specifically human milk oligosaccharides, and *B. infantis* has been investigated demonstrating that carbohydrates are key binding structures for these bacteria and play a critical role in their metabolism and population establishment (92). Identification of these two cell-signaling surface receptors and their subsequent potential extra-cellular glycosylation sites point strongly that these two proteins may be the binding partners of *B. infantis*. Furthermore, even if these two proteins prove not to be the actual binding partners but instead the carbohydrates are, an entire group of known signaling proteins that do not have known ligands are now suspect as binding partners for not only *B. infantis* but other bacteria as well. While presence of these GPCRs is observed in the host cell, the exact sub cellular location of these receptors in this cell line is unknown. Also, while the total amount of GPR20 remains fairly constant across treatment levels and time, GPR161 is only observed in the *B. infantis* treated cells. This could indicate that while initially bound to gangliosides, which are concentrated on GPCRs, *B. infants* then binds GPR20 which then acts as the primary receptor initiating the signaling cascade, resulting in increased

expression of GPR161. As there is no known ligand for GPR161, it is possible that *B. infantis* adhesion induces the expression of this protein with the express purpose of using it to further establish itself on the host cellular surface. As the gut epithelial cells have basolateral and apical surfaces, to be the binding partner of the bacteria the proteins must be expressed on the apical surface. Further investigation of these signaling proteins as to their potential as binding partners of *B. infantis* while very likely, and was therefore tested with additional experimentation. The results form these experiments showed that neither of these induced GPCRs was the binding site of *B. infantis* (Fig. 13).

In conclusion, this study demonstrated that adhesion of *B. infantis* to gut epithelial cells induced significantly higher survival as compared to control cells. Further investigation as to the underlying mechanisms behind this increase survival revealed that gangliosides are used for adhesion, while no protein was identified, in spite induction of two orphan g-protein coupled receptors. Signals transduced via PYK2 to AKT and ultimately to three anti-apoptotic factors. In total, B. infantis reduced cytotoxicity via AKT activation with dual phosphorylation.

CHAPTER V

SUMMARY

Hypothesis:

Mammalian signal transduction via GPCR signaling instigated by adhesion of pathogenic bacteria leads to host cell signaling related to apoptosis, while of probiotic bacteria induces host cell signaling related to cell proliferation.

Objectives**:**

1. Adhere probiotic (*Bifidobacterium infantis*) and pathogenic (*Salmonella* ser. Typhimurium LT2) microbes to Caco2 intestinal cells and measure the gene expression of the signal transduction molecules.
2. Conduct bioinformatic analysis of the gene expression changes to indentify the specific molecules for the associated with g-protein signaling.
3. Candidate molecules will be verified using Western blot analysis. No more than 10 proteins will be verified in this objective.

Analysis of the gene expression data as well as the protein expression data demonstrated conclusively that indeed *B. infantis* adhesion leads to a drastically different cellular signal than cells adhered to *Salmonella* ser. Typhimirium. However, the data did not show that *B. infantis* adhesion resulted in proliferation, but instead an increase in cell survival (Fig. 15). It was seen that g-protein coupled receptors were involved in the host cell signaling. In addition to the original objectives, all of which were performed as described, a cytotoxicity

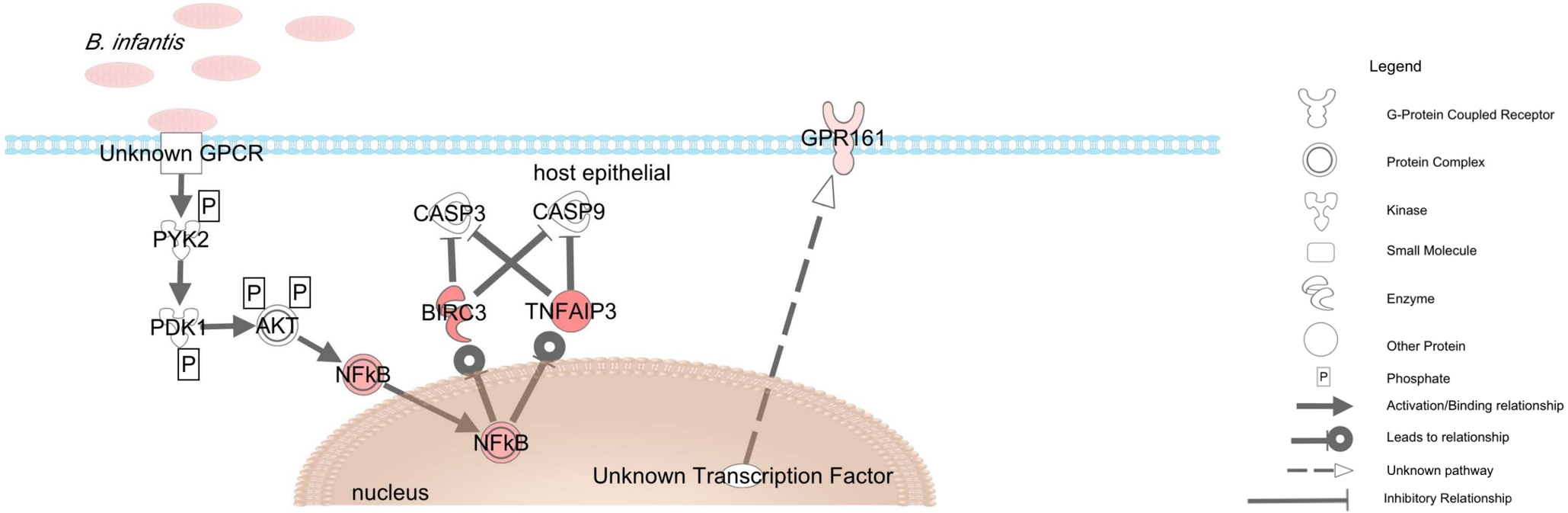

FIG. 15. Proposed model of host reaction to *B. infantis* adhesion. As determined by the data gathered in this experiment, this proposed model shows what effect adhesion of *B. infantis* has on a host epithelial cell. Following adhesion and interaction with some unknown receptor, PYK2 is phosphorylated, leading to the subsequent activation of, also by phosphorylation, PDK1 and AKT. NFKB is the translocated to the nucleus where it is responsible for the induction of the proteins BIRC3 and TNFAIP3. These two proteins have been shown to decrease cell death (10, 28, 150). Via an undetermined pathway the orphan G-protein coupled receptor GPR161 is also induced, as indicated by the red fill, and expressed by the host cell. The significance of the expression of this receptor is not known, although it does not at as the receptor for *B. infantis* adhesion. According to western blot data, the phosphorylation cascade initiated by *B. infantis* adhesion has occurred within 30 minutes post adhesion and by 120 minutes post adhesion the levels of phosphorylated AKT have dropped. Production of BIRC3, TNFAIP3 and GPR161 were all observed within 30 minutes

assay and ganglioside adhesion assay were performed to understand the underlying mechanisms responsible for the induction of the survival genes. These additional studies shed much needed light on this study. Due to the lack of evidence from the gene expression correlating with cell proliferation, as well as the decreased cytotoxicity that was observed, the original hypothesis was set aside and a pursuit of the signals responsible for the decreased cytotoxicity was undertaken. We were successful in uncovering proteins responsible for the increase in cell survival and the signals perpetuated resulting in the increased translation of those proteins.

Overview of Results

A human epithelial model, when adhered to by a known probiotic *B. infantis* displayed significant differences in the survival of those cells. Gene expression analysis implicated that three host genes, BIRC3, TNFAIP3 and SERPINB9 were induced in the host cells and likely the cause of the impact to cell survival seen in the cytotoxicity assay. The mechanism by which these genes were induced was predicted, tested, and proved. Adhesion to host cell gangliosides results in a signaling cascade initiated by unknown GPCRs at the cell surface resulted in the complete activation of AKT and subsequent increased expression of the genes and translation of these survival proteins.

Impact of Work

For the first time, a set of experiments has been conducted that will allow researchers to better assess the properties of bacteria that qualify them as a probiotic. Additionally, a model by which other bacterial species can be tested for their beneficial properties has been established that removes a great degree of complications that arise from animal or human subject systems. While these more advanced models still have a place in this field, I posit that it is a great benefit to gain insights and direction in a reduced variable system before engaging in a complicated and expensive adult animal model.

The next point that should be addressed is that it appears that the word commensal simply does not completely characterize the actual mode of interaction between host and non-pathogenic microbe. *B. infantis* was first placed in this group of neutral organisms, when clearly it is not a neutral organism (Fig. 16). This work furthers the notion that the bacteria residing in the human gut are either doing our bodies some harm, or are doing them some good. While the pathogens have received the bulk of the scientific attention through the centuries, these commensals, if studied more in depth, may prove to not be merely sharing our table, but actively participating in the preservation of the host in which they reside.

Future Work

Identification of the host receptor that is bound by *B. infantis* should be the focus of future work (Fig. 16). While these data suggested that GPR20 and GPR161 were likely involved in binding, the adhesion assay performed to assess

that showed otherwise. The role of these two receptors in the host response should also be evaluated. As this study identified a cell-signaling pathway that was activated by the presence of *B. infantis* what other signaling pathways are active due to the presence of these two cell surface receptors? What impact, if any, do those pathways have on the health of the host? Answering these questions would bring valuable information to the field and

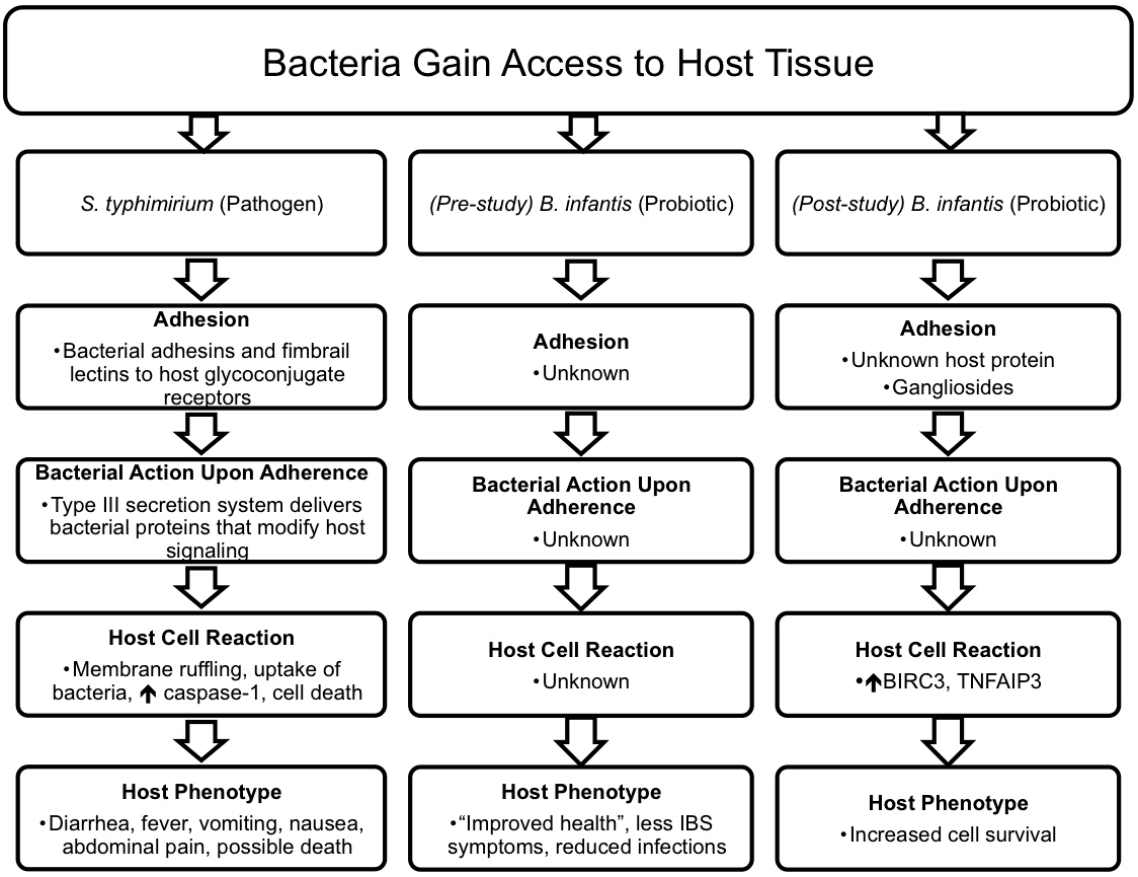

FIG. 16. Additions to field resulting from current study

further clarify the relationship between host and probiotic.

In summary, *B. infantis* adhesion to host cells lead to increased cell survival via cell signaling initiated by g-protein coupled receptors, through complete activation of AKT, and culminating in the translation of three proteins with described impacts on cell survival

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APPENDICES

Appendix A. TABLE A.1. Complete list of significantly differentially expressed host genes following adhesion of *B. infanits*

Symbol	Annotation	Location	Type(s)	Log Ratio
CCL20	chemokine (C-C motif) ligand 20	Extracellular Space	cytokine	-0.32
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	Extracellular Space	cytokine	0.079
CXCL2	chemokine (C-X-C motif) ligand 2	Extracellular Space	cytokine	-0.402
CXCL3	chemokine (C-X-C motif) ligand 3	Extracellular Space	cytokine	-0.015
IL6	interleukin 6 (interferon, beta 2)	Extracellular Space	cytokine	-0.234
IL8	interleukin 8	Extracellular Space	cytokine	0.062
AGPAT4	1-acylglycerol-3-phosphate O-acyltransferase 4 (lysophosphatidic acid acyltransferase, delta)	Cytoplasm	enzyme	-0.406
ARL5B	ADP-ribosylation factor-like 5B	unknown	enzyme	-0.369
BIRC3	baculoviral IAP repeat-containing 3	Cytoplasm	enzyme	-0.243
CES4	carboxylesterase 4-like	Cytoplasm	enzyme	-0.634
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	Cytoplasm	enzyme	-0.34
CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	Cytoplasm	enzyme	-0.274
DLST	dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)	Cytoplasm	enzyme	-0.616
G3BP1	GTPase activating protein (SH3 domain) binding protein 1	Nucleus	enzyme	0.489

Symbol	Annotation	Location	Type(s)	Log Ratio
GEM	GTP binding protein overexpressed in skeletal muscle	Plasma Membrane	enzyme	0.215
HMGCS1	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	Cytoplasm	enzyme	-0.215
LYZ	lysozyme (renal amyloidosis)	Extracellular Space	enzyme	-0.888
MAGT1	magnesium transporter 1	Extracellular Space	enzyme	-1.112
MGST1	microsomal glutathione S-transferase 1	Cytoplasm	enzyme	-0.527
OPA1	optic atrophy 1 (autosomal dominant)	Cytoplasm	enzyme	-0.847
RAB2A	RAB2A, member RAS oncogene family	Cytoplasm	enzyme	-1.12
RAB9A	RAB9A, member RAS oncogene family	Cytoplasm	enzyme	-0.68
RHEB	Ras homolog enriched in brain	Plasma Membrane	enzyme	-1.24
RHOV	ras homolog gene family, member V	Plasma Membrane	enzyme	-0.062
RND1	Rho family GTPase 1	Cytoplasm	enzyme	-0.171
SCD	stearoyl-CoA desaturase (delta-9-desaturase)	Cytoplasm	enzyme	-0.887
SIAE	sialic acid acetylesterase	Cytoplasm	enzyme	-3.026
SOD2	superoxide dismutase 2, mitochondrial	Cytoplasm	enzyme	-1.274
TXN	thioredoxin	Cytoplasm	enzyme	-1.496
UGCG	UDP-glucose ceramide glucosyltransferase	Cytoplasm	enzyme	-0.342
GPR109B	G protein-coupled receptor 109B	Plasma Membrane	G-protein coupled receptor	-0.224
GPR161	G protein-coupled receptor 161	Plasma Membrane	G-protein coupled	-0.61

			receptor	
Symbol	Annotation	Location	Type(s)	Log Ratio
GPR20	G protein-coupled receptor 20	Plasma Membrane	G-protein coupled receptor	-0.485
AREG	amphiregulin	Extracellular Space	growth factor	0.334
EREG	epiregulin	Extracellular Space	growth factor	0.347
JAG1	jagged 1 (Alagille syndrome)	Extracellular Space	growth factor	-0.488
LATS2	LATS, large tumor suppressor, homolog 2 (Drosophila)	Nucleus	kinase	0.016
MAP3K7	mitogen-activated protein kinase kinase kinase 7	Cytoplasm	kinase	-0.308
MAP3K8	mitogen-activated protein kinase kinase kinase 8	Cytoplasm	kinase	-0.764
NUAK2	NUAK family, SNF1-like kinase, 2	unknown	kinase	0.177
NUCKS1	nuclear casein kinase and cyclin-dependent kinase substrate 1	Nucleus	kinase	-1.993
PCK1	phosphoenolpyruvate carboxykinase 1 (soluble)	Cytoplasm	kinase	0.344
PDK4	pyruvate dehydrogenase kinase, isozyme 4	Cytoplasm	kinase	0.004
PIM1	pim-1 oncogene	Cytoplasm	kinase	-0.03
RIOK1	RIO kinase 1 (yeast)	unknown	kinase	-0.479
STYK1	serine/threonine/tyrosine kinase 1	Cytoplasm	kinase	-0.831
NR4A1	nuclear receptor subfamily 4, group A, member 1	Nucleus	ligand-dependent nuclear receptor	0.707

Symbol	Annotation	Location	Type(s)	Log Ratio
NR4A2	nuclear receptor subfamily 4, group A, member 2	Nucleus	ligand-dependent nuclear receptor	-0.125
NR4A3	nuclear receptor subfamily 4, group A, member 3	Nucleus	ligand-dependent nuclear receptor	0.156
NR4A3	nuclear receptor subfamily 4, group A, member 3	Nucleus	ligand-dependent nuclear receptor	-0.01
ALCAM	activated leukocyte cell adhesion molecule	Plasma Membrane	other	-0.613
APP	amyloid beta (A4) precursor protein	Plasma Membrane	other	-0.122
AREGB	amphiregulin B	unknown	other	0.029
BRP44L	brain protein 44-like	Cytoplasm	other	-0.272
C10ORF110	chromosome 10 open reading frame 110	unknown	other	-0.721
C11ORF1	chromosome 11 open reading frame 1	Nucleus	other	-1.343
C17ORF69	chromosome 17 open reading frame 69	unknown	other	-0.225
C2ORF56	chromosome 2 open reading frame 56	unknown	other	-0.836
C6ORF62	chromosome 6 open reading frame 62	unknown	other	-1.095
CALD1	caldesmon 1	Cytoplasm	other	-0.389
CALD1	caldesmon 1	Cytoplasm	other	-0.179

CALD1	caldesmon 1	Cytoplasm	other	-0.544
Symbol	Annotation	Location	Type(s)	Log Ratio
CARD10	caspase recruitment domain family, member 10	Cytoplasm	other	-0.413
CCNI	cyclin I	unknown	other	-0.621
CCNL1	cyclin L1	Nucleus	other	-0.446
CD9	CD9 molecule	Plasma Membrane	other	-0.079
CDC37L1	cell division cycle 37 homolog (S. cerevisiae)-like 1	Cytoplasm	other	-0.212
CHMP4B	chromatin modifying protein 4B	Cytoplasm	other	-1.19
COL1A2	collagen, type I, alpha 2	Extracellular Space	other	0.245
CYR61	cysteine-rich, angiogenic inducer, 61	Extracellular Space	other	-0.115
CYR61	cysteine-rich, angiogenic inducer, 61	Extracellular Space	other	0.067
DCAF5	DDB1 and CUL4 associated factor 5	unknown	other	-0.545
DDIT4	DNA-damage-inducible transcript 4	Cytoplasm	other	0.088
DLC1	deleted in liver cancer 1	Cytoplasm	other	-1.214
DNAL1	dynein, axonemal, light chain 1	unknown	other	-1.065
DST	dystonin	Plasma Membrane	other	0.342
EFNA1	ephrin-A1	Plasma Membrane	other	-0.222
EMP1	epithelial membrane protein 1	Plasma Membrane	other	0.024
FAM134 B	family with sequence similarity 134, member B	unknown	other	-0.667
FBXO45	F-box protein 45	unknown	other	0.026
FCF1 (includes EG:51077)	FCF1 small subunit (SSU) processome component homolog (S. cerevisiae)	unknown	other	-1.268

Symbol	Annotation	Location	Type(s)	Log Ratio
FLJ10357	hypothetical protein FLJ10357	unknown	other	-0.22
FLJ36848	hypothetical LOC647115	unknown	other	-1.371
GADD45A	growth arrest and DNA-damage-inducible, alpha	Nucleus	other	0.242
GADD45B	growth arrest and DNA-damage-inducible, beta	Cytoplasm	other	-0.072
GRAMD2	GRAM domain containing 2	unknown	other	-0.746
HABP4	hyaluronan binding protein 4	Cytoplasm	other	-0.244
HHLA2	HERV-H LTR-associating 2	unknown	other	-0.575
HINT3	histidine triad nucleotide binding protein 3	unknown	other	-1.756
HOOK1	hook homolog 1 (Drosophila)	Cytoplasm	other	-1.049
HSP90B1	heat shock protein 90kDa beta (Grp94), member 1	Cytoplasm	other	-1.22
HSPA4L	heat shock 70kDa protein 4-like	Cytoplasm	other	-0.53
IER3	immediate early response 3	Cytoplasm	other	-0.573
IER3IP1	immediate early response 3 interacting protein 1	unknown	other	-0.945
IER5	immediate early response 5	unknown	other	-0.308
IER5L	immediate early response 5-like	unknown	other	0.185
INPP5F	inositol polyphosphate-5-phosphatase F	unknown	other	-0.713
ISG15	ISG15 ubiquitin-like modifier	Extracellular Space	other	-0.316

Symbol	Annotation	Location	Type(s)	Log Ratio
ITPRIPL2	inositol 1,4,5-triphosphate receptor interacting protein-like 2	unknown	other	-0.947
KLHDC7A	kelch domain containing 7A	unknown	other	0.2
LOC100128893	hypothetical protein LOC100128893	unknown	other	0.201
LOC100130360	hypothetical LOC100130360	unknown	other	-0.11
LOC100292959	similar to hCG2042049	unknown	other	-0.997
LOC284454	hypothetical protein LOC284454	unknown	other	0.203
LOC285628	hypothetical protein LOC285628	unknown	other	-0.078
LONRF3	LON peptidase N-terminal domain and ring finger 3	unknown	other	-0.456
MEX3A	mex-3 homolog A (C. elegans)	unknown	other	-0.283
MGC16275	hypothetical protein MGC16275	unknown	other	-0.755
MLLT4	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 4	Nucleus	other	-0.903
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	Cytoplasm	other	-0.211
PDLIM3	PDZ and LIM domain 3	Cytoplasm	other	-0.49
PDLIM3	PDZ and LIM domain 3	Cytoplasm	other	-0.017

PEAR1	platelet endothelial aggregation receptor 1	unknown	other	-0.408
Symbol	Annotation	Location	Type(s)	Log Ratio
PER1	period homolog 1 (Drosophila)	Nucleus	other	0.319
PLIN2	perilipin 2	Plasma Membrane	other	-0.366
PSME4	proteasome (prosome, macropain) activator subunit 4	unknown	other	-0.012
RGMA	RGM domain family, member A	Plasma Membrane	other	-0.395
RP5-1022P6.2	hypothetical protein KIAA1434	unknown	other	-0.061
RPL27A	ribosomal protein L27a	Nucleus	other	-1.232
RPL38 (includes EG:6169)	ribosomal protein L38	Cytoplasm	other	-1.016
RPS11	ribosomal protein S11	Cytoplasm	other	-0.828
RTN4	reticulon 4	Cytoplasm	other	-0.226
S100P	S100 calcium binding protein P	Cytoplasm	other	-0.512
SERPINB9	serpin peptidase inhibitor, clade B (ovalbumin), member 9	Cytoplasm	other	-0.851
SNCA	synuclein, alpha (non A4 component of amyloid precursor)	Cytoplasm	other	-0.728
SOCS3	suppressor of cytokine signaling 3	Cytoplasm	other	0.044
SPATA2L	spermatogenesis associated 2-like	unknown	other	-0.793
SPATS2L	spermatogenesis associated, serine-rich 2-like	unknown	other	0.121
SPATS2L	spermatogenesis associated, serine-rich 2-like	unknown	other	0.117

SPON1	spondin 1, extracellular matrix protein	Extracellular Space	other	-0.627
SPRED1	sprouty-related, EVH1 domain containing 1	Plasma Membrane	other	-0.054
Symbol	Annotation	Location	Type(s)	Log Ratio
SSX2IP	synovial sarcoma, X breakpoint 2 interacting protein	Plasma Membrane	other	-0.754
TAGLN	transgelin	Cytoplasm	other	-0.629
TIPARP	TCDD-inducible poly(ADP-ribose) polymerase	unknown	other	-0.122
TM4SF1	transmembrane 4 L six family member 1	Plasma Membrane	other	-0.517
TMEM206	transmembrane protein 206	unknown	other	-0.852
TMEM49	transmembrane protein 49	Plasma Membrane	other	-0.314
TMEM49	transmembrane protein 49	Plasma Membrane	other	-0.137
TMTC2	transmembrane and tetratricopeptide repeat containing 2	Cytoplasm	other	-0.293
TNFAIP3	tumor necrosis factor, alpha-induced protein 3	Nucleus	other	0.152
TNFAIP3	tumor necrosis factor, alpha-induced protein 3	Nucleus	other	0.065
TPM3	tropomyosin 3	Cytoplasm	other	-0.64
TRAF3IP3	TRAF3 interacting protein 3	unknown	other	-0.367
WHSC1	Wolf-Hirschhorn syndrome candidate 1	Nucleus	other	-0.346
ZNF295	zinc finger protein 295	Nucleus	other	-0.312
PLAU	plasminogen activator, urokinase	Extracellular Space	peptidase	-0.173

PSMB4	proteasome (prosome, macropain) subunit, beta type, 4	Cytoplasm	peptidase	-0.676
SPG7	spastic paraplegia 7 (pure and complicated autosomal recessive)	Cytoplasm	peptidase	-0.688
Symbol	Annotation	Location	Type(s)	Log Ratio
DUSP4	dual specificity phosphatase 4	Nucleus	phosphatase	-0.105
PPP3CC	protein phosphatase 3 (formerly 2B), catalytic subunit, gamma isoform	unknown	phosphatase	-0.503
ATF3	activating transcription factor 3	Nucleus	transcription regulator	-0.298
ATF3	activating transcription factor 3	Nucleus	transcription regulator	-0.063
BHLHE40	basic helix-loop-helix family, member e40	Nucleus	transcription regulator	0.49
BTG2	BTG family, member 2	Nucleus	transcription regulator	-0.062
CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	Nucleus	transcription regulator	0.037
CREM	cAMP responsive element modulator	Nucleus	transcription regulator	-0.01
CREM	cAMP responsive element modulator	Nucleus	transcription regulator	-0.231

CREM	cAMP responsive element modulator	Nucleus	transcription regulator	-0.514
CREM	cAMP responsive element modulator	Nucleus	transcription regulator	-0.353
Symbol	Annotation	Location	Type(s)	Log Ratio
CSRNP1	cysteine-serine-rich nuclear protein 1	Nucleus	transcription regulator	-0.212
CYLD	cylindromatosis (turban tumor syndrome)	Nucleus	transcription regulator	-0.461
DLX1	distal-less homeobox 1	Nucleus	transcription regulator	-0.336
EGR3	early growth response 3	Nucleus	transcription regulator	-0.004
ELF3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	Nucleus	transcription regulator	0.286
ELF3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	Nucleus	transcription regulator	0.278
ELF3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	Nucleus	transcription regulator	0.381
ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	Nucleus	transcription regulator	-0.1
GRLF1	glucocorticoid receptor DNA binding factor 1	Nucleus	transcript	-0.842

			ion regulator	
HEY1	hairy/enhancer-of-split related with YRPW motif 1	Nucleus	transcription regulator	-0.23
Symbol	Annotation	Location	Type(s)	Log Ratio
HOXA2	homeobox A2	Nucleus	transcription regulator	0.193
HOXA2	homeobox A2	Nucleus	transcription regulator	-0.101
IRF1	interferon regulatory factor 1	Nucleus	transcription regulator	-0.236
IRF8	interferon regulatory factor 8	Nucleus	transcription regulator	-0.34
JUN	jun oncogene	Nucleus	transcription regulator	0.713
JUNB	jun B proto-oncogene	Nucleus	transcription regulator	-0.316
KLF6	Kruppel-like factor 6	Nucleus	transcription regulator	-0.113
KLF7	Kruppel-like factor 7 (ubiquitous)	Nucleus	transcription regulator	-0.401
LMCD1	LIM and cysteine-rich domains 1	Cytoplasm	transcript	-0.463

			ion regulator	
LMCD1	LIM and cysteine-rich domains 1	Cytoplasm	transcription regulator	-0.02
Symbol	Annotation	Location	Type(s)	Log Ratio
LMO1	LIM domain only 1 (rhombotin 1)	Nucleus	transcription regulator	-0.456
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	Nucleus	transcription regulator	0.26
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	Nucleus	transcription regulator	-0.419
MBD2	methyl-CpG binding domain protein 2	Nucleus	transcription regulator	-0.341
NFIL3	nuclear factor, interleukin 3 regulated	Nucleus	transcription regulator	0.182
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	Nucleus	transcription regulator	0.415
NFKBIE	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	Nucleus	transcription regulator	-0.357
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	Nucleus	transcription regulator	-0.454
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	Nucleus	transcription regulator	0.539

RCAN1	regulator of calcineurin 1	Nucleus	transcription regulator	0.108
Symbol	Annotation	Location	Type(s)	Log Ratio
RCAN1	regulator of calcineurin 1	Nucleus	transcription regulator	-0.919
REL	v-rel reticuloendotheliosis viral oncogene homolog (avian)	Nucleus	transcription regulator	0.012
SCML1 (includes EG:6322)	sex comb on midleg-like 1 (Drosophila)	Nucleus	transcription regulator	-0.49
SERTAD 2	SERTA domain containing 2	Cytoplasm	transcription regulator	-0.051
SOX7	SRY (sex determining region Y)-box 7	Nucleus	transcription regulator	0.325
SPEN	spen homolog, transcriptional regulator (Drosophila)	Nucleus	transcription regulator	-1.554
TLE1	transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)	Nucleus	transcription regulator	-0.434
TSC22D1	TSC22 domain family, member 1	Nucleus	transcription regulator	-0.234
ZNF397	zinc finger protein 397	Nucleus	transcription regulator	-0.796

ZNF689	zinc finger protein 689	Nucleus	transcription regulator	-0.042
Symbol	Annotation	Location	Type(s)	Log Ratio
EEF1D	eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein)	Cytoplasm	translation regulator	-1.006
EIF5B	eukaryotic translation initiation factor 5B	Cytoplasm	translation regulator	-0.945
ALB	albumin	Extracellular Space	transporter	-1.771
AP2B1	adaptor-related protein complex 2, beta 1 subunit	Cytoplasm	transporter	-0.598
ATP6V0E1	ATPase, H+ transporting, lysosomal 9kDa, V0 subunit e1	Cytoplasm	transporter	-1.295
FABP1	fatty acid binding protein 1, liver	Cytoplasm	transporter	-1.28
GLTP	glycolipid transfer protein	Cytoplasm	transporter	-0.865
SLC25A25	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 25	Cytoplasm	transporter	-0.179
VPS13A	vacuolar protein sorting 13 homolog A (S. cerevisiae)	Cytoplasm	transporter	0.027

APPENDIX B. GO Trees

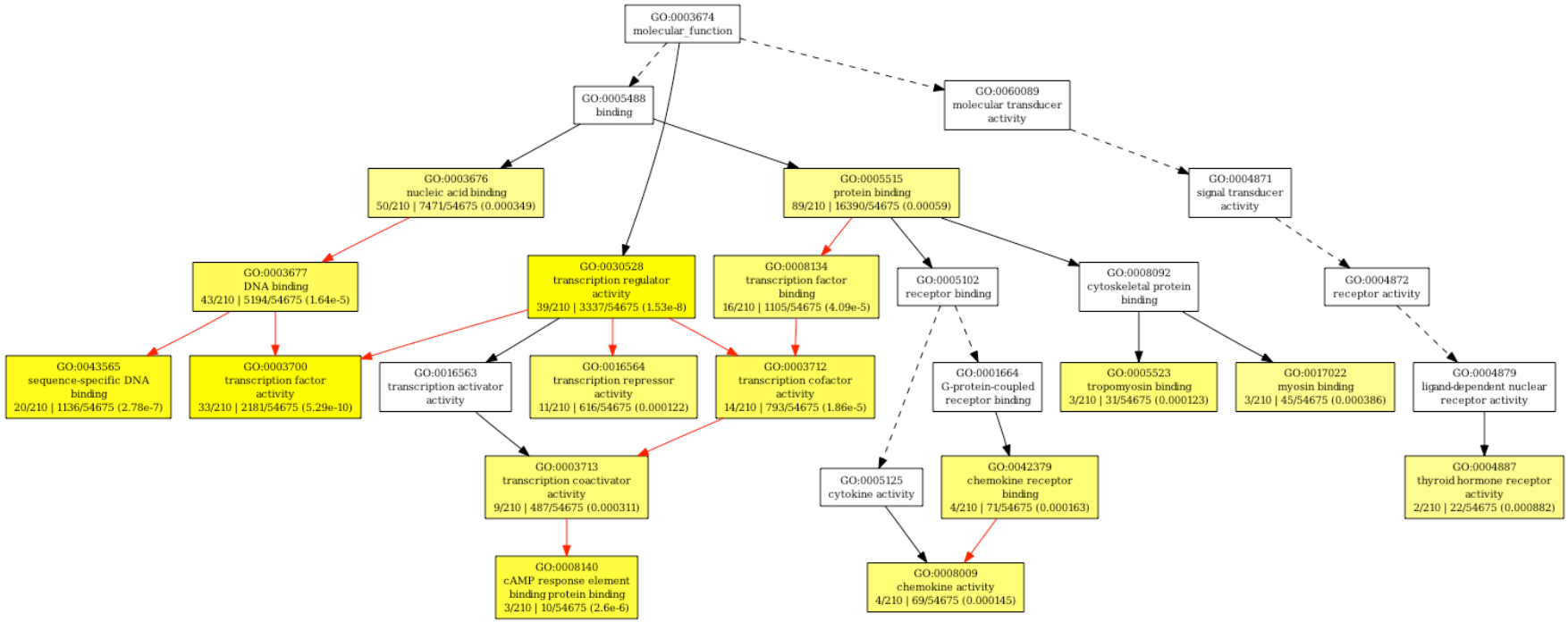


FIG. B.1. GO Tree of Molecular Function

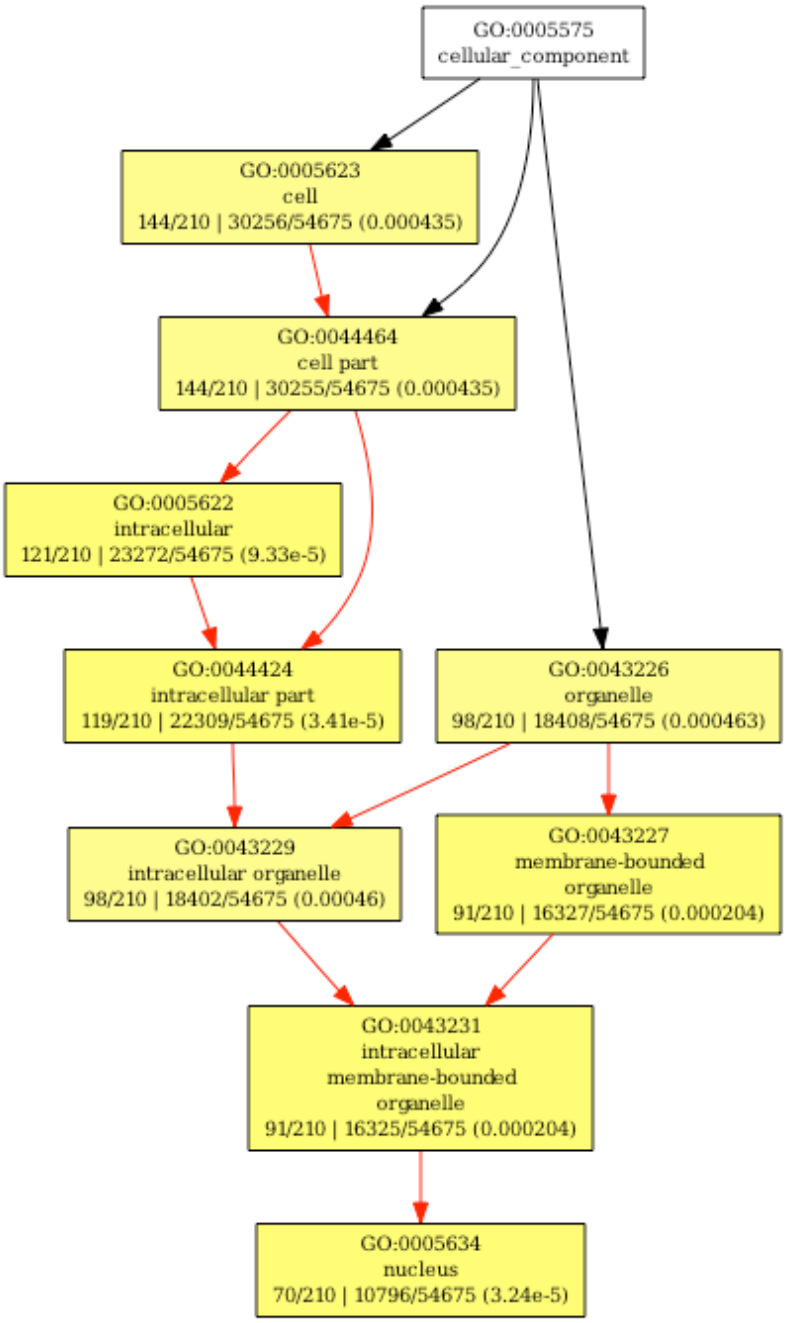
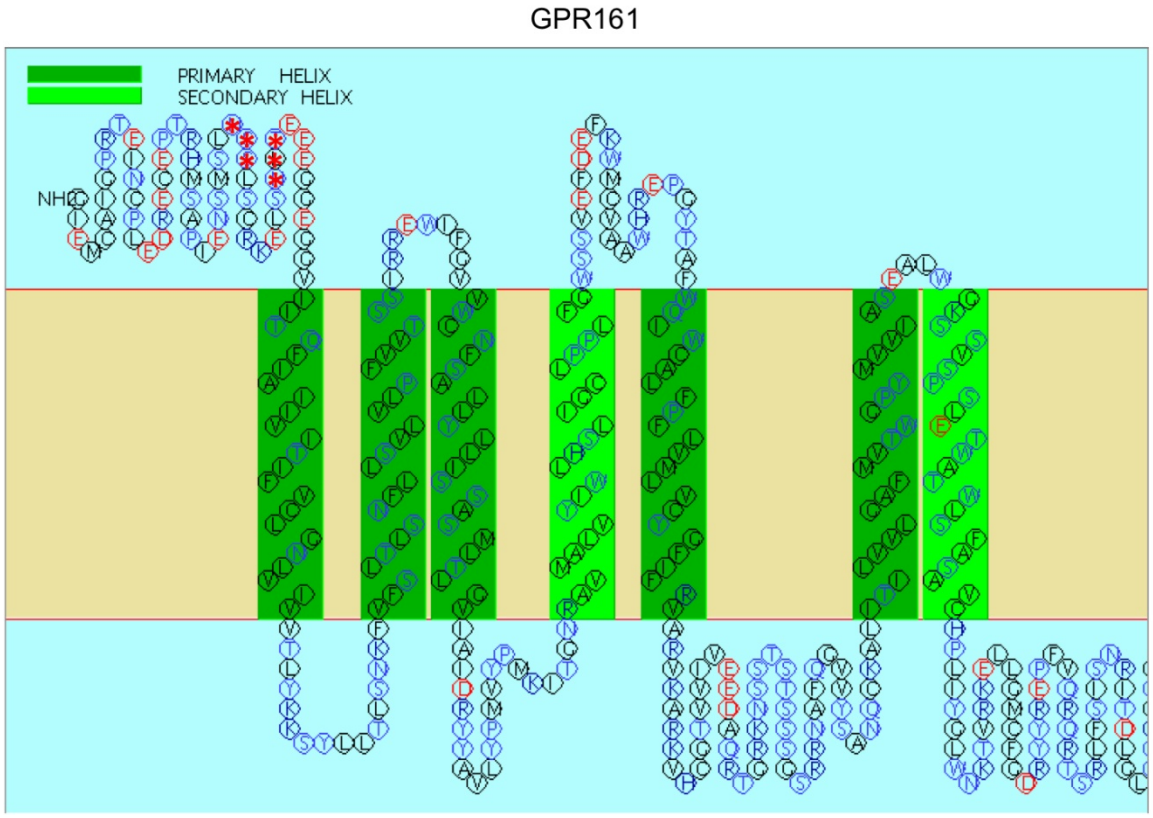


FIG. B.3. GO Tree of Cellular Component

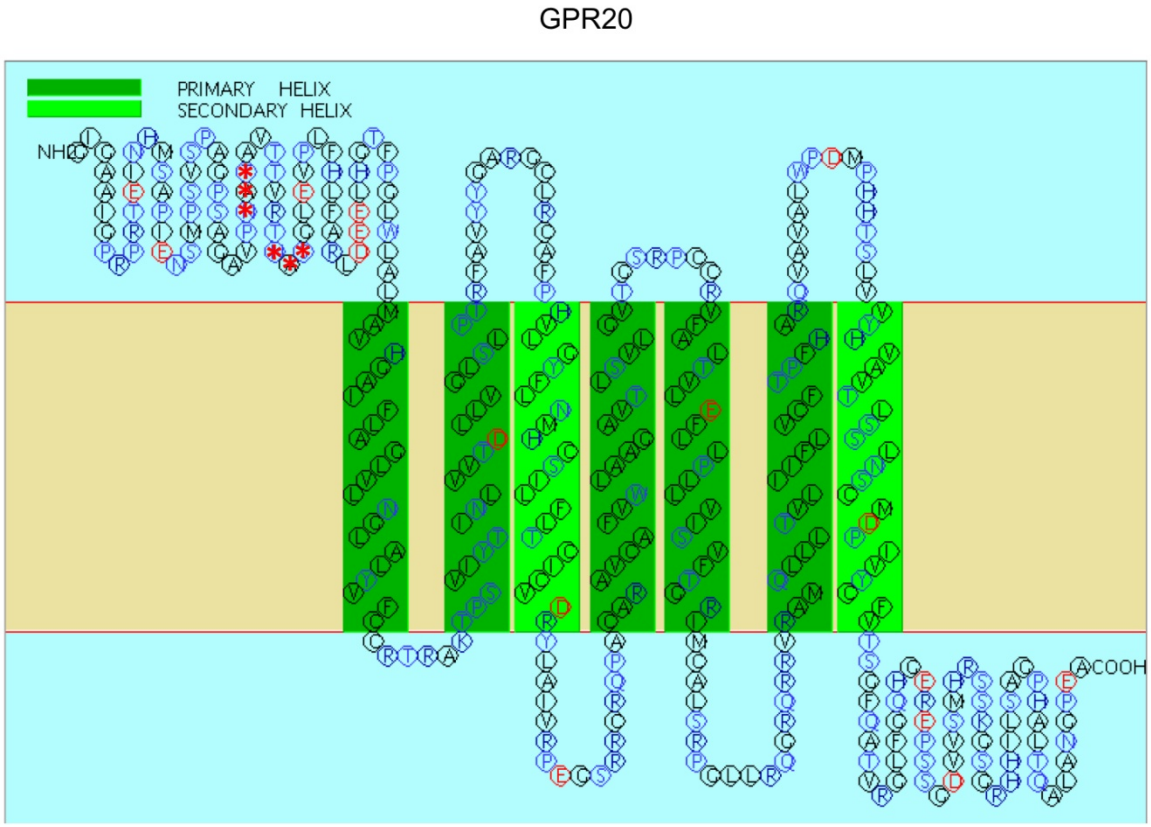
APPENDIX C. GPCR Protein Analsysis
A)



FIGS. C.1.-C.2. GPR20 and GPR161 protein analysis

A) GPR161 transmembrane regions (http://bp.nuap.nagoya-u.ac.jp/sosui/cgi-bin/adv_sosui.cgi) and N-linked glycosylation sites (http://www.cbs.dtu.dk/services/NetNGlyc/) as predicted based on sequence. Red asterisks indicate predicted location of extra-cellular N-linked glycosylation sites. Tan area indicates membrane of cell, while uppermost blue field represents the extra-cellular space, with the lower blue field the intra-cellular space.

B)



B) Similar plot as shown in A), for GPR20.

APPENDIX D. Permission Letter from P. Desai

10/13/2009

Reed Gann
Center for Integrated BioSystems
Utah State University
4700 Old Main Hill
Logan UT, 84322-4700
(435)795-3356

Dear Prerak Desai,

I am in the process of preparing my thesis in the Biology Department at Utah State University. I hope to complete it in October 2009.

I am requesting your permission to include the attached material as shown. I will include acknowledgments and/or appropriate citations to your work as shown and copyright and reprint rights information in a special appendix. The bibliographical citation will appear at the end of the manuscript as shown. Please advise me of any changes you require.

Please indicate your approval of this request by signing in the space provided, attaching any other form or instruction necessary to confirm permission. If you charge a reprint fee for use of your material, please indicate that as well. If you have any questions, please call me at the number above. I hope you will be able to reply immediately. If you are not the copyright holder, please forward my request to the appropriate person or institution.

Thank you for your cooperation,

Reed Gann

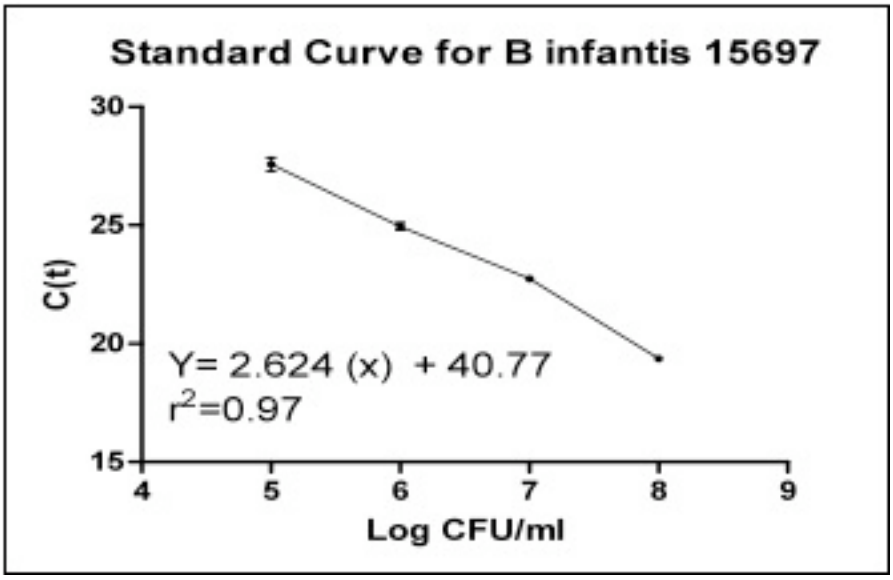

Standard Curve for B infantis 15697
30
25
20
15
C(t)
Y= 2.624 (x) + 40.77
r2=0.97
4
5
6
7
8
9
Log CFU/ml

Fig. B.2. GO tree of biological process